

Winning a nuclear test-ban by stealth?

The US Congress has been teasing the administration by tagging a nuclear test-ban onto a domestic spending bill. The trick will help to advertise an important question, but the need now is for serious and energetic diplomacy.

THE US Congress played a sharp trick on the administration by attaching to a bill that funds energy and water projects — including the Superconducting Super Collider in Texas — a requirement that there should be no tests of nuclear weapons in the United States for a period of nine months until the middle of next summer, and that there should be a permanent test-ban beginning in 1996. Last week, President George Bush reluctantly signed the bill, although he called the test ban “highly objectionable” and promised Congress that he would try to reverse it.

The Congress has done well to make it plain that the issue of the test-ban cannot indefinitely be allowed to stay at the back of the world’s consciousness. Indeed, it is high time that the question was given the attention it will now be given. One reason is that, in just over two years, the signatories of the Nuclear Non-Proliferation Treaty (NPT) will either have to affirm that they are satisfied with the treaty, or the arrangement will collapse. The meeting of the signatories in 1995 will therefore differ radically from the review conferences hitherto held at intervals of five years, which have mostly been rancorous affairs. Too much rancour next time, and there will be no treaty.

Catastrophe

That would be a catastrophe. It is unthinkable that the decades after 1995 will be peaceable decades without something like the NPT. That is not to suggest that the treaty is a leak-proof instrument. On the contrary, as the case of Iraq has shown all too vividly, it is entirely feasible for governments to sign the NPT, to accept the routine inspections that entails and then to busy themselves with schemes for developing warlike nuclear facilities on the side. But that is only half the story. Iraq, if not a member of the NPT, would no doubt have been able to get ahead even more quickly with its clandestine project. But most members of the treaty have signed because they have no nuclear ambitions of a military character, and seek such benefits as there may be from the general knowledge of that state of grace. Sceptics should remark that South Africa, now anxious to win general respect, is one of the more recent recruits to the treaty. Its weaknesses notwithstanding, the NPT has been a powerful influence against the proliferation of nuclear weapons.

But the NPT was never meant to be a sufficient safeguard against the proliferation of nuclear weapons. Soon after it was signed, it was found necessary to supplement it with an agreement among the potential suppliers of nuclear materials and equipment; that agreement, known as the London agreement, needs to be supplemented by sanctions against suppliers who break the rules. But the most powerful influence against the spread of nuclear weapons to countries that have elected to stay outside the treaty has been political pressure from more powerful allies and associates. It is inevitable that that should be so. And political pressure (mixed with inducements) can be effective — witness the recent compliance of North Korea with the non-proliferation regime. But political pressure will serve the cause of non-proliferation only if the number of candidates against which it must be directed is comparatively small. Perhaps the chief function of the NPT is to make the black sheep conspicuous.

Survival

So what must be done to increase the chances that the NPT will survive beyond 1995? The present treaty commits the nuclear powers to negotiate away their nuclear forces. At the conference in 1995, they will indeed be able to boast that there are now agreements between the United States and Russia (the voluntary successor of the Soviet Union on arms control) to limit their strategic weapons and weapons of intermediate range in Europe.

But that will not still all dissent. Non-nuclear powers at previous review conferences have repeatedly and vociferously complained that the major nuclear powers continue to make more sophisticated nuclear weapons within the framework of whatever agreements they have reached with each other. That is why the re-emergence of the test-ban question is timely as well as important: nothing could be a better proof, in 1995, of the willingness of existing nuclear powers to sign a test-ban treaty. At one stroke, that would rob dissenters of the argument (for which they cannot be blamed) that the nuclear powers seek the compliance of other members of the treaty with rules more rigorous than they will themselves accept.

Winning a comprehensive test-ban agreement, perhaps as part of an amended NPT, will not be easy. Two nuclear powers, China and France, are not members of the NPT (although they have agreed informally to behave as



if they were). If, as seems likely, a comprehensive test-ban agreement would be the best way of ensuring the continuation of the NPT, the two years left before the 1995 conference are hardly time enough. But would it not be a great misfortune if the NPT fell apart for lack of the time in which to make its continuation likely? That is one good reason why the US Congress has done the world a public service by advertising the importance of the test-ban issue.

Unfortunately, there is still more to be done before 1995. The past few years have shown all too well that the tendency towards nuclear proliferation has not melted away. Who can be sure, for example, that neither Iran (a signatory of the NPT since the Shah's time) nor Pakistan (not a signatory) is following along the road pioneered by Iraq? As things are now, even compliance by India (a conspicuous non-signatory) would not necessarily secure that of Pakistan, which may (like Iran) believe it has a duty towards the Muslim Middle East, already polarized by Israel's cryptonuclear status. And what is to be made of the ex-Soviet republics other than Russia? The Russian government claims to be the inheritor of the Soviet Union's international obligations, but is it in a position to assure its partners of the compliance of its lesser neighbours, Khazakstan and the Ukraine, for example? It would be a great surprise if it could speak for even the physical integrity of nuclear weapons and unprocessed materials.

That is why there is no time to waste in preparing for the NPT conference in 1995, the year after next, which, by the standards of diplomacy, may be likened to the day after tomorrow. There is endless persuasion of recalcitrant nuclear powers to be done, not to mention technical work on the treaty itself and on the safeguards regime required for a future now likely to be very different from what seemed possible a few years ago. (The case of Iraq has shown that the safeguards regime should ideally concentrate on the most suspicious countries, however inequalities that may seem.) It is a great waste of scarce resources that the inspection agency, the International Atomic Energy Agency in Vienna, should deal on an equal footing with, say, Sweden and Khazakhstan. Proliferators would cry "discrimination" if the present rules were changed, but that is a revealing cry.

There remain over-arching questions such as whether non-compliance with an amended NPT would justify expulsion from the United Nations. That question, prompted by recent moves towards enforcement in the UN Security Council, will surely be answered "No" unless there is a test-ban. Meanwhile, the most urgent need is for the non-signatory nuclear powers, France and China, to be brought into the fold. It is ridiculous that they stand aside from a treaty they say they abide by in all except name. It helps a little that France has recently been making eyes at Britain on co-ordination of nuclear policy. Britain, with hardly any other international influence left, should seize the chance for the common good. □

Megaprojects galore

Europe seems keen on construction projects with benefits for the environment: will environmentalists agree?

EVEN if the European enterprise is temporarily under a cloud of scepticism, even disbelief, Europe's physical monuments to its hopes of closer union appear to be multiplying fast. Last week, the waterway joining the Danube with the Rhine was opened for traffic, more than a millennium after people in Europe first started talking about some such scheme. At the same time, the Swiss population has voted in favour of an ingenious, if costly, way of keeping heavy traffic off rural Alpine roads; Switzerland will now probably sign a treaty with the European Communities (EC) giving it exemption from Europe's highway standards in return for a means of transporting trucks beneath the Alps on flatcars. Both tunnels, beneath the Gotthard and the Lötschberg, should be in place early next decade. The Swiss tunnels, the longer of which is 50 kilometres long, are comparable in scale with the tunnel beneath the English Channel (otherwise La Manche), which should be operating within a year.

Interestingly, all three schemes have been partly inspired by environmental considerations, and in particular by the wish to transfer the transport of goods from overstressed road networks. The link between the Rhine and Danube waterway systems will have overt and substantial economic benefits as well. All three will also allow charges for the costs of transport to be levied directly on the beneficiaries, which is a sensible long-standing goal. The projects are therefore striking illustrations of how improvements of the environment can be brought about by civil engineers, whose construction schemes are nevertheless opposed as almost as a matter of routine by those professing care for the quality of the environment. It is a question of some importance whether Europe's environmental organizations, coloured various shades of green, can be persuaded to give Europe's future megaprojects the welcome they deserve.

There are a lot of them ahead. The EC, urged on by the SNCF (the French railway) has been dreaming of a network of fast railways throughout its own region and penetrating far into the east. These projects will inevitably require land now occupied by houses and other unrelated structures. The evolution of this European railway network will be a fierce test of the green lobby's willingness to put the greater good ahead of narrow sectional interests. It has not, so far, done well; in the case of the British proposal to build a high-speed railway link between the landfall of the Channel tunnel (called Eurotunnel), environmental interests in South-East England have so far forced revisions of the preferred route which are so costly that, in present economic circumstances, it is unaffordable. It is to be hoped that Europe as a whole will be more deliberately level-headed. □

Gene wars escalate as US official battles NIH over pursuit of patent

Washington. A leading US government lawyer has blocked the National Institutes of Health (NIH) from continuing to pursue patents on thousands of partial cDNA sequences, a controversial — and by some accounts illegal — move that reflects a bitter battle within the government. The decision by Michael Astrue, general counsel for the Department of Health and Human Services (HHS), NIH's parent agency, is intended to thwart Bernadine Healy, the NIH director, and reflects the views of critics of gene patenting, including much of the biotechnology industry.

Last week, Astrue told HHS officials that he would not permit NIH to respond to the US Patent and Trademark Office's rejection on 20 August of its application. NIH had intended to answer the patent office's decision within the next several months, and Healy told Congress last month that her legal experts believed that the agency could win its case (see *Nature* 359, 263; 1992).

Astrue's tactics shows how divisive the gene patent issue has become and the lengths to which opponents are willing to go to undermine the NIH position. At one point, the patent office became so frustrated with conflicting instructions from warring factions within the government that it warned the government that it would henceforth communicate only with NIH's law firm, Knobbe, Martens, Olson & Bear of Newport Beach, California.

The leading critic of NIH's patent application within the Bush administration, Astrue believes that gene sequences with unknown functions should not be patented because such patents would hinder companies from developing gene-based products. Over the past several months, he has tried repeatedly to prevail against the objections of Healy and, in the latest case, even his own boss, Louis Sullivan, the HHS secretary.

Before the patent office had even completed its initial review this summer, Astrue asked it to suspend consideration of the application on policy grounds. In a letter of 18 June to Harry Manbeck, the US patent commissioner, Astrue said that "I have concluded that [the NIH applications] do not satisfy the threshold legal requirements for an invention because they do not describe the use or function of the sequences." NIH officials — who were not told of Astrue's letter before it was sent — protested that his request was an attempt to sabotage the application.

They need not have worried. The patent

office, arguing that the case was too important to drop, rejected Astrue's request. In a letter to Astrue on 25 June, patent officials said that "the strong public interest in identifying and resolving any issue of patentability presented in these applications" demanded their prompt consideration. Manbeck warned Astrue that his "apparent conclusion that some, as yet unidentified,

from handling biotechnology issues for one year, he plans to resume his campaign at the first opportunity in the course of representing industrial clients as a member of a Boston-based law firm. Astrue, contacted last week, declined to comment on the issue.

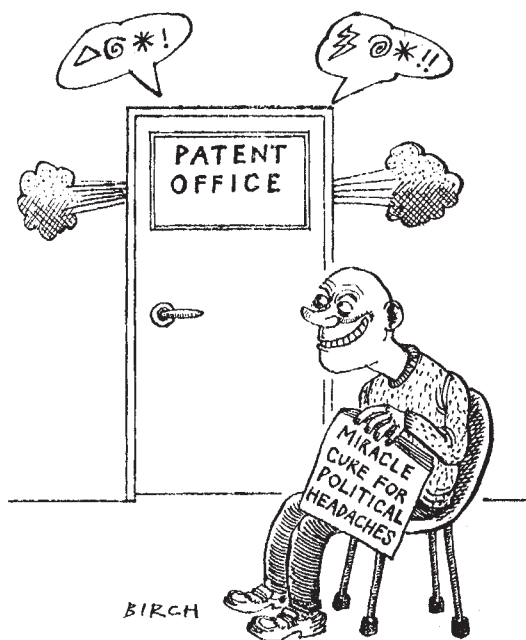
Despite his imminent departure, Astrue believes that his arguments will prevail, telling associates that he has the support of officials at OMB and the White House Council on Competitiveness, which under Vice President Dan Quayle has become a powerful mechanism for industry to influence government policy (see *Nature* 353, 198; 1991). A spokesman for the Council said that it had no policy on gene patents and would not confirm that it had discussed the issue with Astrue.

But just how Astrue can defy his own boss is not clear. Sullivan supports Healy on this issue and last month allowed NIH to file for patents on additional cDNA partial sequences. "Mike Astrue is not Secretary Sullivan; he's just one part of this", says a spokesman for the department. Astrue is claiming that the department is required to take the advice of its ranking legal authority, arguing that NIH made its application in "bad faith" and that pursuing it would constitute legal malpractice.

Richard Godown, the president of the Industrial Biotechnology Association (IBA), says it would be "very unusual" for Sullivan or the acting HHS general counsel to overrule Astrue's decision after he leaves. Nor would Godown want them to. Although the IBA backed NIH's original filing, Godown says that was done only to prevent individual NIH scientists from applying for the patents privately, something IBA felt would set an even worse precedent. Godown hopes that the last word on the matter — the patent office's interim rejection — will stand unchallenged and will discourage further attempts to patent unknown genes.

Whatever the resolution of the legal questions, it is not clear what is to be gained by blocking NIH from pursuing its patent bid. Private companies and individuals are rumoured to have filed for similar gene patents, and they are not likely to abandon their applications even if NIH does. The patent office itself has asked to take the subject through a full exchange of arguments to its conclusion, so as to leave a final decision as precedent. The US government's internal battle over gene patents may produce casualties, but it seems unlikely to end the war.

Christopher Anderson



portion of the applications does not meet the legal threshold for a patentable invention" was simply inappropriate. If Astrue had additional information — rather than simply an opinion — on the patent claim, he should have submitted it, the patent official scolded.

The rebuke did not deter Astrue. After NIH's application was refused in August, he ordered the agency not to release or discuss the arguments made by the patent office. When Healy requested permission to bring up the subject in congressional testimony last month, Astrue and the Office of Management and Budget (OMB) deleted references to the decision from her written testimony. Healy nevertheless defied the order and discussed the issue in her oral testimony, but it was a risky move that may have hurt her politically.

Some observers attribute Astrue's fervour to both his personal beliefs and the fact that he will be leaving the government next month for a job in which a reputation for gene-patent busting may serve him well. Although he is prohibited by federal law

Reshuffling of funding councils jeopardizes plans for UK academic computer network

London. The forthcoming realignment of Britain's higher education funding councils into four regional bodies has thrown a wrench into the works for plans to develop Super JANET, a successor to the existing British academic computer network.

Super JANET is a high band-width optical fibre network capable of sending information at a rate of gigabits a second — a thousand times faster than JANET. Whereas JANET is capable of carrying data, text files and electronic mail, Super JANET will allow distributed computing, transfer of still and moving images, interactive use of computers and immediate visual representation of data and the remote use of computers with differing architectures for single tasks. As such, Super JANET is expected to improve access to computing facilities, reducing delays and reversing the trend of some researchers being forced to work overseas to obtain access to more powerful US networks.

The Universities Funding Council (UFC) has promised to spend £20 million (US\$35 million) to develop Super JANET between 1993 and 1996. But from April next year, responsibility for funding higher education moves from it and the Polytechnics and Colleges Funding Council (PCFC) to four regional funding councils, covering England, Wales, Scotland and Northern Ireland. The dissolution of the UFC means that plans for Super JANET must be renegotiated with the new funding bodies.

Also at stake is the future management structure of British academic networks. There is a proposal to transfer, by April 1993,

JANET and Super JANET from the funding council's Information Systems Committee (ISC) to the UK Education and Research Networking Association (UKERNA), an independent body with the nonprofit-making status of a scientific research association. The networking association will draw funds from the new higher education funding bodies, the research councils and possibly the Department of Trade and Industry.

However, the people behind UKERNA are having trouble convincing the Department For Education to provide them with funding, and they are worried that UKERNA may be left out of the hierarchy if the situation is not resolved before the funding systems change. Although the department apparently supports the UKERNA proposal, its civil servants have tied the elected management in a seemingly endless cycle of question-and-answer over the last six months. UKERNA hopes to break this deadlock with a report from an independent consultant on UKERNA's policy, plans and possible contribution to be submitted to the department and to Treasury by November.

In the interim, the association will spend the rest of the £5 million it was allocated in 1992 to hire a contractor to set up a pilot network. The aim is to secure additional funding by demonstrating real applications to ministers in early 1993. If the money is not forthcoming this year, attempts will be made to keep the project alive until 1994.

UKERNA would also like to set up a trading company that enables industrial companies to tap into JANET and, eventually,

Super JANET. In the same way that Super JANET is intended to improve access to distant academic resources, adding industry should facilitate collaborations. Initially, only companies with active collaborations with university groups will be allowed to join, and no more than 20 per cent of UKERNA's income is supposed to come from industry to ensure that the network continues to serve academics.

Ian Mundell

Russians reject plan for elite research centres

Moscow. The Russian Academy of Sciences has rejected a proposal by the government to pool its research funds and create a network of well-funded scientific centres. The proposal was intended to ensure that a core of world-class researchers receive sufficient funding to maintain their laboratories even in the face of the current economic crisis. But academicians, after five hours of heated debate, withheld their support out of concern that such a shift in funding would cripple existing institutes and jeopardize their status.

The idea was presented by Boris Saltykov, minister of science, to the academy's presidium at a meeting on 15 September. Institutes designed as federal centres would receive modern equipment, a steady flow of current publications and the opportunity to travel regularly.

It would cost an estimated 1 million rubles to support the work of each researcher at a federal centre, with the money coming from reallocating the present budget. Saltykov believes that several thousand researchers could be supported at that level without robbing the rest of the scientific community of needed resources.

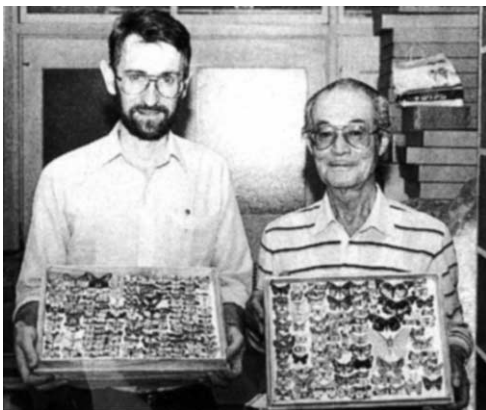
The proposal grows out of a recognition by the scientific community that Russia can no longer afford a system characterized by huge institutions and low scientific productivity. However, the presidium's vote demonstrates that change will not come easily. Its opposition reflects a feeling that such a parallel system of centres may undermine the academy's authority, if not its reputation.

At the same time, most academicians acknowledge that some new structure is needed. One compromise may be to allow the academy to take the lead, designating institutes to be selected as centres and deciding what privileges they will be granted.

Vladimir Pokrowsky

Japanese moth collection lands in London

The Natural History Museum in London has taken delivery of 200,000 moth specimens in a bequest from the Japanese entomologist Hiroshi Inoue (right). The collection, which includes more than 500 holotype specimens (the basis of initial species classification), is the result of nearly half a century of field work in Japan, Taiwan and Nepal. Inoue wanted the collection moved before his death from its present home in Otsuma Women's University, Saitama Prefecture, so that he could continue to work on it in collaboration with museum entomologists. Malcolm Scoble (left) says that the collection is an outstanding scientific resource, documenting sites that no longer exist in pristine condition. A trust fund is planned to allow young Japanese researchers to study at the museum.



Ian Mundell

Europe tightens rules that govern homeopathic products

Munich. The European Parliament has tightened its supervision of homeopathic products to bring them into line with a new system that will permit the free movement of medicaments within the European Communities (EC).

The move modifies last year's controversial directive, which would have exempted all products claimed to be homeopathic from the strict regulations applied to the licensing of conventional pharmaceuticals. Under the new procedures only products of very high dilution (1 in 10^4) will qualify for the proposed simplified registration. No claims for special medical indications will be allowed.

However, the change is not sufficient to satisfy the European Federation of Pharmacology Societies, which says that the directive lends credibility to homeopathy by referring to very dilute preparations as homeopathic 'medicines'. Fearful of the increasing power of the alternative-medicine lobby in Europe, the federation would like to substitute the more neutral term 'homeopathic preparations'.

With or without the amendment, EC member states have less than a year to introduce the directive into their national legislation; full harmonization of licensing laws for medicaments is to be achieved by 1995. This means that some countries, such as Greece, will have to change laws that forbid the import of homeopathic products while others, in which such products are heavily promoted, will have to tone down claims for homeopathic 'remedies'.

Interest in homeopathy varies widely, with an estimated 40 per cent of the French, 18 per cent of Belgians and 10 per cent of Italians using homeopathic products regularly. On the other hand, homeopathy is rarely practised in Denmark.

Control of the products also varies widely: in Portugal, Norway and Belgium, for example, doctors are free to prescribe homeopathic products, while the Netherlands this year introduced legislation, in line with the EC directive, prohibiting any claim for a specific therapeutic indication.

Pharmacologists are also concerned about the growing pressures to incorporate homeopathy and other unconventional medical practices into mainstream medicine. Several health insurance companies offer policies

covering alternative medicine, and alternative practices are beginning to influence training and research.

In Germany, where 22,000 homeopathic products are registered using criteria less rigorous than those applied to its 34,000 conventional drugs, medical schools are now obliged to test students on their knowl-

edge of alternative medicine. Organizations that had previously refused to cooperate are now beginning to work more closely with the centralized examination board in selecting questions.

In Switzerland, the national funding agency

set aside SFr6 million (US\$4.5 million) in 1990 for a five-year programme of research into alternative medicine. At the same time, two Swiss practitioners of alternative medicine received support from the European Commission to coordinate the results of European research projects, which may or may not receive public funding. When Finland challenged the approach as "unscientific", a compromise was reached and an 'unconventional medicine project' was created under the direction of Klaus von Berlepsch, deputy director of clinical research at Hoffmann-LaRoche in Basel.

Although commission coordination ('COST') programmes do not provide funding for research — instead, they pay for travel and publications — the programme estimates that ECU21 million (US\$27 million) is likely to be spent on national research projects in the next five years. In the United States, the National Institutes of Health have been ordered by Congress to begin a \$2-million-a-year programme to assess the value of unconventional medical practices (see *Nature* 358, 5; 1992).

Although the European Federation of Pharmacology Societies acknowledges the popularity of homeopathy in parts of Europe and the inherent safety of products of very high dilution, it does not want the European Parliament to appear to endorse the unproven belief that homeopathic preparations may be beneficial. This is particularly important at a time when such countries as Hungary, Czechoslovakia and Russia are seeing a surge in popularity of cheap alternative medicines.

Alison Abbott

Royal Society wants extra powers for science office

London. The Royal Society has proposed that the Office of Science and Technology (OST) should be given a budget separate from the annual allocation for science to address Britain's international science commitments and oversee domestic research issues. The recommendations come in the society's report, *The Future of the Science Base**, which was delayed because of the creation of OST earlier this year.

Although only a modest amount is proposed — £100 million (US\$180 million) to oversee domestic research and a similar amount to meet the country's international subscriptions (£105.8 million for 1992–93) — the Royal Society feels that the money could have a considerable effect. "It would help the UK gain a substantially better return from European Communities research, in particular, and international science more generally, and better promote science and technology within the UK", the report says. The OST is considering the idea.

International subscriptions are usually paid in currencies other than sterling, and fluctuations in the exchange rates can heavily influence the real cost to the research councils. Having OST take responsibility for these funds would free the research councils from unexpected drains on their budgets and make planning more secure.

The move also acknowledges the political element in international collaborations and makes government accountable for any influence it might exert. Researchers complain that they have sometimes been unable to exercise scientific judgements — even to the extent of pulling out of collaborations — because of objections from the Foreign Office and other government departments.

On top of the subscriptions, which would be transferred from the science vote to the OST budget, the Royal Society proposes that new money should be found to address issues vital to the national research and development infrastructure that transcend individual departmental responsibilities (biotechnology and biodiversity are examples), and to match research programmes supported by the European Communities (EC).

Britain is unusual among European countries in that research budgets are reduced by the amount of money obtained from the EC: elsewhere, the funds are considered additional. As well as being a disincentive to EC collaboration, the policy means that researchers working with EC money are progressively drawn away from British research priorities.

Ian Mundell

* 11.50, Publication Sales, Royal Society, 6 Carlton House Terrace, London SW1Y 5AG, telephone 071-839-5561).

UK moves closer to free market for research funding

London. Government and industrial laboratories would be allowed to compete for funding from the British research councils under a new proposal from the Advisory Board for the Research Councils. The councils would also be encouraged to pool their resources to support worthwhile research in biotechnology.

The seven-man subcommittee that produced the report* included Sir Mark Richmond, chairman of the Science and Engineering Research Council; Tom Blundell, secretary of the Agricultural and Food Research Council; Dai Rees, secretary of the Medical Research Council; and Eileen Buttle, secretary of the Natural Environment Research Council. Such senior backing from within the research councils practically ensures that the proposal will be implemented. It also has the support of the science minister, William Waldegrave, who says that "it is entirely consistent with our policy on opening up an internal market for government-funded research and development".

The report would overturn a long-standing policy that each research council should stick to its own area, although the charters of each council do not specify where

the research they fund should be carried out. Some administrative hurdles must also be cleared; the SERC, for example, hopes to change its rules to allow for external bids.

The opening of research council funds to proposals from industrial and government laboratories is likely to upset university researchers, who fear that money will be siphoned off for applied research. "Funds for basic research are so thin anyway that it is madness to dilute them further by giving them to people to do near-market research", says Derek Roberts, provost of University College, London. Research funds are already expected to be strained by applications from the new universities — until this year the polytechnics — many of which have said they intend to do more basic research. On the other hand, the report suggests that academics have access to other sources of government and industrial funding, although few think this will happen.

The government laboratories have welcomed the move but are not saying to what extent they will take advantage of this new funding source. Many have become agencies, which makes them responsible for generating their own income. Although this

makes them more predatory, it also raises the costs of research as there is no government subsidy for wages and overhead. Without a good deal of creative accounting, the universities and research council institutes will be able to give better value for money.

Explicit funding by more than one council is permitted but not widespread. It has happened most easily where the mission-orientated research councils have shown an interest in molecular biology. However, most academics acknowledge that a proposal to a single council can be broadened if it contains research attractive to a larger audience.

As the title of the proposal suggests, the decision to admit government and industrial laboratories is particularly important for biotechnology research, a subject in which all the research councils and several industries have an interest. Attempts to coordinate biotechnology over the past decade have been frustrated by a failure to agree on inter-council programmes, and the freeing of funding channels is expected to strengthen the current coordinating body, the Biotechnology Joint Advisory Board.

Ian Mundell

* Report of the ABRC Biotechnology Subcommittee, Advisory Board for the Research Councils, 5B3 Sanctuary Buildings, Great Smith Street, London SW1P 3BT, telephone 071-925-5966.

Small businesses to get bigger slice of US research pie

Washington. The US Congress last week agreed to increase the share of federal research dollars going to small businesses, part of a government-wide trend towards more funding for research aimed at commercial products. The legislation will more than double the size of the Small Business Innovation Research (SBIR) programme over the next five years, from \$430 million a year to more than \$1 billion by 1997.

Although industrial researchers welcome the increase, university scientists are less enthusiastic. The reason is simple: the money will come from budgets that finance their projects.

The SBIR programme currently gets its funding by taking 1.25 per cent from each federal agency that spends more than \$100 million on research, including the National Institutes of Health, the National Science Foundation and the National Aeronautics and Space Administration. Beginning this month, that share will increase to 1.5 per cent, eventually reaching 2.5 per cent in 1997.

At NSF, for example, that means its SBIR programme will grow this year from \$22 million to about \$27 million and that \$5 million less will be available for traditional grants to academic scientists. NSF officials may not like the involuntary shift in funds, but they say that the programme is too popular to oppose. **Christopher Anderson**

Ludwig estate to support research

Washington. Six US institutions with cancer research programmes have inherited the bulk of the fortune of Daniel K. Ludwig, a reclusive cancer philanthropist who died in

late August at the age of 95 and whose estate was recently estimated at \$1.2 billion. But the known amount — \$46 million — is far below what most would have expected from a man who 20 years ago



Daniel K. Ludwig

created a string of cancer research institutes throughout Europe with a present endowment of \$700 million.

It will be 21 years, in fact, before the institutions mentioned in Ludwig's will — the Massachusetts Institute of Technology, Memorial Sloan-Kettering Cancer Center and the medical schools of Harvard

University, the Johns Hopkins University, Stanford University and the University of Chicago — receive most of Ludwig's bequest, which consists mostly of domestic real estate. The will stipulates that Ludwig's property be placed in the Virginia and D.K. Ludwig Fund for Cancer Research, whose trustees will distribute investment income at their discretion to the six beneficiaries. After 21 years, the remaining money will be divided by the trustees among the six beneficiaries.

Ludwig apparently disposed of most of his fortune in 1971, when he used his considerable European assets to create the Ludwig Institute for Cancer Research. The institute includes 11 branches in eight countries; each branch supports several investigators, who focus on one biological problem associated with cancer.

Ludwig, who gained most of his wealth from shipping, mining and real estate, never suffered from cancer, nor did any of his close relatives, according to his friend and former physician Hugh Butt. Ludwig supported cancer research, Butt says, because it is "the most feared disease and affects children as well as adults".

Traci Watson

Congress supports breast cancer research by taking \$210 million from military

Washington. Last week the US Congress took \$210 million from this year's defence budget and told the government to spend it on breast cancer research. This is the first time that legislators have succeeded in tapping the military budget to pay for a civilian research programme and may represent a down payment on the long-awaited 'peace dividend' following the end of the Cold War. However, it also continues a congressional tradition of designating money for studies on a specific disease, a practice known as 'earmarking' that many believe is a poor way to spend scarce research dollars.

The amendment to a defence spending bill, introduced by Senator Tom Harkin (Democrat, Iowa), removes \$210 million from general defence accounts and gives the money to the US Army for breast cancer research. But the bill also commands the military to enlist the help of other government agencies; in practice, that means the National Cancer Institute (NCI). NCI stewardship "certainly would provide the most efficient mechanism" for spending the money wisely, says Peter Reinecke, a staff member to Harkin.

The 'wall' surrounding defence funds has survived several assaults since it was created by a 1990 agreement on federal spending that established limits on spending for military and civilian programmes.

So why did research on breast cancer, rather than, say, AIDS or Alzheimer's disease, finally make a breach in the wall? One reason is that many male legislators face female opponents in next month's election and want to demonstrate their concern for women's problems. Legislators are especially eager to restore images tarnished by the 1991 Senate confirmation hearings for Supreme Court Justice Clarence Thomas, accused by a former employee of sexual harassment.

Despite the congressional support for research (the amendment passed the Senate by a 89:4 margin), it is not clear how the Office of Management and Budget (OMB) will react. Early last week, OMB said that the research money would be counted as domestic rather than military spending, meaning that a small slice must be taken out of the civilian budgets of each federal agency, including NCI's parent organization, the National Institutes of Health (NIH), with the total equal to the money added for breast cancer research.

Although the final appropriations bill attempts to avoid that step by explicitly defining the research money as a military expenditure, OMB may still decide to cut domestic programmes across the board. But even if a reduction were to be applied throughout the \$9-billion NIH budget, it

would add up to much less than the \$210 million that NCI will gain.

The amendment from Harkin, who has lost two sisters to breast cancer, does not specify how the money should be spent, but NCI already has plenty of ideas. Samuel Broder, the director of NCI, says that his preference would be for peer-reviewed, investigator-initiated basic research. NCI's budget request for the 1994 fiscal year (beginning on 1 October 1993), now under review at OMB, includes such ambitious projects as the development of new cancer therapies and clinical trials of preventative drugs, as well as studies of the link between diet and breast cancer. NCI also wants to establish more of its Specialized Programs of Research Excellence, centres for both basic and clinical research into breast cancer.

In testimony last month before a task force of the Budget Committee in the House of Representatives, Bernadine Healy, the director of NIH, complained about Congress's habit of telling NIH to spend a certain portion of its budget on a specific disease. "We would hope advice would be accompanied by a cheque," Healy said. Now that she has gotten her wish, the NIH director must decide whether cheques accompanied by congressional advice are really a good way to increase the NIH budget.

Traci Watson

Doubts grow faster than budget in new Framework plan

Munich. The European Communities (EC) are sticking to their aim of nearly doubling research spending by 1998 despite dwindling support by member states. In announcing the EC's next five-year ECU14.7 billion (US\$11 billion) budget proposals for research and development — known as the 'Fourth Framework' — research minister Filippo Pandolfi said last week that it was in line with the EC's overall objective of increasing industrial competitiveness by strengthening Europe's science and research base.

Although details are not yet available, Pandolfi says that he wants to spend ECU1 billion to continue training and promoting the mobility of research workers within Europe. He allocates ECU11.6 billion to 'tightly focused programmes of industrial and social importance' to individual countries but more appropriately carried out at a European level. The new plan emphasizes generic technologies with broad industrial applications and multidisciplinary academic research. More environmental and other socially orientated research will be sup-

ported, and a new programme will allow for collaborative research with non-EC countries in Central and Eastern Europe and in the developing world.

However, it is likely that Europe's Council of Ministers, meeting in Britain in December, will ask Pandolfi to trim his request. Research is one of the most vulnerable parts of the overall EC budget for 1993–97, designed by EC president Jacques Delors, and many countries say they cannot afford to do more of it. Delors hoped to increase research spending from 3.7 per cent of public expenditure to 5 per cent by 1998, narrowing the gap in investment between Europe and its chief economic competitors, the United States and Japan.

The fate of the Maastricht Treaty will not influence the size or characteristics of the Fourth Framework, but its adoption may make it harder for member nations to approve particular projects. Under the Single European Act, Framework programmes must be approved unanimously by the Council of Ministers in a four-stage procedure; specific research projects within the framework

must be approved by a qualified majority, again according to a four-stage procedure. Although the Council of Ministers does not need a formal agreement from the European parliament, the two bodies must consult one another. This can lead to ambiguities — for example, disagreements not always directly related to the projects themselves delayed funding of the third Framework for two and a half years.

The Maastricht Treaty would set up even more hurdles. The procedure of 'codecision' — established in the interests of ensuring greater democracy — means that any decision taken by the ministers must be supported by a more powerful European parliament, whose agreement will carry equal weight legally. This process is bound to move slowly. In addition, the Council of Ministers would have to approve each project unanimously; in theory, a single country could block any project. The European Commission, which is responsible for the EC's directives, estimates that it could take three years to make decisions on individual projects once the treaty is ratified. **Alison Abbott**

Apologies to Bianconi

SIR — National science policy has been a primary focus of mine for many years. It is a subject to which I have dedicated considerable time and energy. The issue of overselling science is an issue in national science policy that deserves not only mine but others' time and energy. Over the past year, I have conducted a public debate with editors from *Nature*, *The Scientist* and *Research/Penn State* about overselling science regarding biomimesis and bio-derived materials.

However, as a part of that debate, I am afraid that a colleague of mine, Dr Patricia Bianconi, may have been unfairly caught in the middle, and to the extent that she feels her research has been a victim in this debate, I extend to her this apology, as I never intended her research itself to be the focus of the debate.

In the policy memorandum I privately circulated to various agencies and persons, I used the word "duplicating". The statement was: "this result — duplicating work precipitating very small crystals of any one of a dozen phases including CdS in an inorganic gel . . .". While I believe the work derives from the general experiments done by many on crystallization in gels, Bianconi's work had the special feature that she obtained an organized array of crystals of CdS in an organic host. In this respect her work did not duplicate earlier research and contains novel and unreported findings. The significance of this work will, as in all science, be determined over the course of time. I recognize that some well respected scientists find her results to be quite significant.

It was also imprecise for me to state that Bianconi had not "read or cited" the literature. I had no first-hand knowledge of whether she had or had not read the literature. It was not cited. In large part, the literature to which I had referred was that setting forth the replamine process, published in the 1970s. My criticism of a failure to cite this literature was not aimed at Bianconi's *Nature* article (349, 315–317; 1991 — decisions of whether to cite articles should fairly be decided by authors and reviewers), but at the *Research/Penn State* article, which includes a lengthy text on the biological connections of Bianconi's work, without reference to the biomimetic work at Pennsylvania State University reported in more than 50 papers and 80 patents leading to prosthetic devices as well as

electroceramic composites, which have gone all the way to the marketplace.

My focus was on the use of "biomimetic" or other bio-related terms by her and others in conjunction with the research in question. I never stated nor meant to imply or infer that she was careless, or engaged in lazy practices or cheating of any kind. Similarly, I have never meant to imply, nor do I believe now, that she engaged in any form of scientific misconduct. Finally, I regret that the private memorandum I circulated to funding agencies and others contained the imprecise statements I have referenced above, and that some of these statements were published in the open literature.

Unfortunately this whole affair has taken on an untoward tone. There have been errors, omissions and exaggerations — perhaps on both sides. It is time to close this chapter for the good of the university.

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Animal welfare

SIR — It was, I believe, Professor Thomas Jukes who wrote "To every complex question there is a simple answer — and it's wrong". The recent correspondence on animal welfare demonstrates the truth of his aphorism. Everyone thinks they know what is meant by welfare, but it cannot be measured directly and is extremely hard to define. Ultimately the well-being of an animal is a function of sensory perception and must relate to its physical and mental state; this is governed by a broad range of external factors and in turn influences the animal's behaviour, physiology, growth and production. Although changes can be regarded as indicators of welfare they are, unfortunately, also influenced by a large number of environmental factors.

Egg production in the laying hen, which Jukes clearly regards as a primary indicator of welfare¹, is a good example. It is sensitive to relatively minor changes in variables such as photoperiod, ambient temperature and dietary energy content, none of which has much impact on welfare. One can, therefore, equate changes in egg production to welfare only if all other influential factors have been controlled. It follows that it is not possible to compare the egg production of hens in battery cages with that in some other husbandry system and infer that differences between them tell one anything about the relative states of

welfare of the hens in the two systems. Still less can one argue, as Jukes appears to be doing^{1,4}, that if production reaches some target value which is commercially satisfactory, it demonstrates that welfare is also satisfactory.

However, if production is measured within an experiment where all the main factors are controlled, and the treatments at issue are systematically varied, then information is obtained from which valid conclusions can be drawn. Such experiments, reviewed by Hughes³, demonstrate that the egg output of caged hens declines progressively as the number of hens per cage is increased and also as the space per hen is reduced. These two effects are independent and additive. On the basis of this criterion, then, to optimize the welfare of battery hens they should be given as much space as possible, while the numbers of hens in each cage should be reduced to a minimum. This can be regarded as no more than a starting point. If, as much research suggests¹, hens need a nest site, have feet ill-adapted to standing for long periods on sloping wire floors and benefit from access to substrate for foraging and scratching, then far more radical design changes to housing systems are required to safeguard welfare. But that is another question.

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NASA on a tether

SIR — As an amateur sailor, I can guess what happened in the NASA tether experiment (*Nature* **358**, 526 & 529; 1992). When the satellite was released from the Shuttle and the tether unwound, it went slack and a 'riding [overlapping] turn' developed. Any seaman who winds in or pays out cable on a rotating capstan knows from bitter experience that he must control the cable to avoid this condition.

When the gravitational potential difference between the two vehicles applied a tension to the cable, the riding turn locked the other turns on the cable drum.

No marks to NASA, which should have asked a crane driver for advice.

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Correction

The following reference, containing articles amplifying the point made in the letter by Josephson *et al.* (*Nature* **358**, 618; 1992), was omitted from the reference list appended to that letter: *Frontier Perspectives*, Vol. 3, No. 1 (Center for Frontier Sciences, Temple University, Philadelphia, 1992). □

Myths of the German atom bomb

Mark Walker

The persistent controversy surrounding the role of the German scientists in developing an atomic bomb during the Second World War can be laid to rest by the release of the Farm Hall transcripts.

WHY is the controversy surrounding the "German atomic bomb" — the efforts during the Second World War to harness the economic and military applications of nuclear fission — so persistent¹? This controversy began at a time when little was known about what German scientists may have done in the service of National Socialism. Although we now have a thorough picture of nuclear fission under Hitler (see, for example, refs 2,3), many of the myths that plague this history have not been dispelled.

Most of the debate revolves around two brief episodes that have very little to do with the actual research on nuclear reactors and weapons: the visit paid by Werner Heisenberg and Carl Friedrich von Weizsäcker in 1941 to their Danish mentor Niels Bohr in occupied Copenhagen⁴; and the imprisonment of ten German scientists in a country house named Farm Hall in Godmanchester near Cambridge in the United Kingdom. Here I shall examine the second episode.

The main reason why the legends surrounding 'Hitler's bomb' cannot be dispelled is that they have always been used — at least in part — as a vehicle to debate a fundamental, but often repressed dilemma of contemporary science: should scientists work for their country or for their own professional advancement no matter what? Copenhagen and Farm Hall have been used and misused as proxies, red herrings and straw men in this debate.

The German atomic bomb

The question of whether German scientists tried to make atomic bombs has no one answer: if to try means investing the huge amounts of money, manpower and materials that were clearly necessary to develop and manufacture nuclear weapons, then neither German scientists nor anyone else in Germany 'tried'. But if to try means efforts to create and analyse substances known to be nuclear explosives as quickly as possible and in increasing amounts without harming the German war effort, then Germany, and these scientists, did try (see ref. 2).

The German work can be best put into perspective by comparing it to the joint US – UK effort. Up until the winter of 1941–42, the German work ran practically parallel to the Allied research. When the responsible science policy makers in the United States and Germany re-

viewed their respective nuclear-fission projects, both sides had comparable scientific results. But the context of the war was decisively different for both sides. In Germany, with a strained war economy and the recent failure of the *Blitzkrieg* in the (then) Soviet Union, it was commonly assumed that if Germany could not win the war quickly, it would lose. In the United States, with an untouched economy and vast resources, it was assumed that it would take four or five years to wear down Germany. Thus whereas Vannevar Bush and his colleagues in Washington decided that atomic bombs could influence the outcome of the war and therefore must be attempted, Erich Schumann and other officials at German Army Ordnance decided that nuclear

Heisenberg, Horst Korsching, Max von Laue, Carl Friedrich von Weizsäcker and Karl Wirtz. His selections were sometimes arbitrary: several scientists who were released knew as much if not more than most of their colleagues at Farm Hall and others, like von Laue, were brought along merely because Goudsmit thought that they would make good spokesmen for German science.

What has made the situation of unusual interest is the fact that the UK authorities installed secret microphones for eavesdropping on their conversations. In 1962 Leslie Groves, the head of the Manhattan project, used excerpts from transcripts of these conversations to spice up his immodest memoirs, thereby revealing their existence⁶. Ever since, scientists and other interested parties have tried to force the release of these transcripts.

Why have these conversations been considered so important? Obviously because it was assumed on all sides that only by eavesdropping on what these scientists said in reaction to the news of Hiroshima could we learn the 'truth' about the German atomic bomb. But which truth has been sought? Groves' memoirs and his selective reproduction of excerpts from the Farm Hall transcripts have moulded the terms of the debate. His brief quotations suggest that Heisenberg did not understand the difference between an atomic bomb and a nuclear reactor, and that after the shock of Hiroshima, von Weizsäcker disingenuously created the story that the German scientists had not made nuclear weapons because they had not wished to.

Almost everyone interested in the mysterious Farm Hall transcripts has sought to find in them evidence for their side of the debate: either that Heisenberg was incompetent and von Weizsäcker therefore mendacious, or that Heisenberg knew how to make an atomic bomb and von Weizsäcker was just being honest. But this superficial concern with scientific competence has been part of a different, much deeper debate, the 'myth of the German atomic bomb'. One argument is that because of moral concerns, these German scientists consciously chose to control, slow down and divert their research to deny nuclear weapons to Hitler; the other view is that these scientists failed to achieve a nuclear weapon because of incompetence,

IMAGE
UNAVAILABLE
FOR COPYRIGHT
REASONS

National Archives, Washington, DC.

Farm Hall — home to a myth?

weapons were irrelevant to the outcome of the war (page 49 of ref. 2). The effect of these different decisions was immediate. Although German scientists continued research into all applications of nuclear fission, including nuclear explosives, by the end of the war they had managed to match only what the Allies had accomplished by June 1942.

But it was not so clear in the autumn of 1944 that the threat of German atomic bombs was merely a bad dream. The 'Alsos mission', a special scientific intelligence-gathering unit, followed closely on the heels of the advancing Allied armies and searched for any evidence of Nazi nuclear weapons. The scientific head of the mission, Samuel Goudsmit, had to decide whether documents and materials discovered should be seized or destroyed, and whether any scientists found should be arrested⁵.

Goudsmit decided that ten scientists should be detained, including Erich Bagge, Kurt Diebner, Walther Gerlach, Otto Hahn, Paul Harteck, Werner

but if they had succeeded, they would have handed these weapons to the Nazi leadership so that Germany could win the war (pages 184–186 of ref. 1).

Operation Epsilon

In November 1991, Nicholas Kurti, Rudolf Peierls and Margaret Gowing wrote a letter to the British Lord Chancellor, also signed by about 20 other distinguished scientists and historians, calling for the release of the Farm Hall recordings. A few months later, the Public Record Office in London released 212 pages of transcripts from 'Operation Epsilon', the code name for the eavesdropping. Shortly thereafter, the National Archives in Washington, DC also released their copy, which is much easier to read and includes a few dozen pages that are not in the British document.

What does Operation Epsilon tell us? There are no revelations or surprises concerning the German efforts to harness nuclear fission, but the conversations are interesting and historically significant in other ways. The scientists detained at Farm Hall were concerned with four questions: whether they were Nazis; whether they knew how to make atomic bombs; whether they wanted to make the bombs; and their future.

In answer to the first question, the two scientists who had been in the Nazi party were understandably defensive about their political past, and their colleagues were not always understanding. The transcripts show that several of them seemed concerned to rid themselves of any connection to National Socialism, prompting their jailors to wonder whether they had guilty consciences. But there was more to being a Nazi than party membership. According to the transcripts, the wardens at Farm Hall detected what they believed was the lingering effect of National Socialist ideology: with the possible exception of von Laue, the Germans apparently still believed in the master race.

The scientists expressed different opinions about the worst abuses by the Germans under National Socialism. Karl Wirtz was one of the few to state flatly that 'we' Germans had committed crimes. But when they learned of the detonation of the US atomic bomb at Hiroshima, this problem practically vanished. Other questions were now more important.

When Goudsmit asserted after the war that Heisenberg did not understand how an atomic bomb worked, there were three parts to this alleged lack of understanding. First, Heisenberg did not realize that plutonium was fissionable material suitable for a nuclear explosive; second, he did not realize that nuclear weapons used fast-neutron chain reactions; and third he did not understand

that only relatively small amounts of fissionable material, not tons, were needed (page 204 of ref. 2).

There is ample evidence, available before the release of the Farm Hall recordings, that Heisenberg understood that uranium 235 and plutonium were fissionable materials suitable for nuclear explosives and that such nuclear explosives used fast-neutron chain reactions (page 56 of ref. 2). The transcripts corroborate Heisenberg's understanding and have cleared up the last remaining matter, that of critical mass.

According to the transcripts, Heisenberg reacted to the news of Hiroshima with great scepticism and indeed argued at first that the Allies could not have separated out the hundreds of tons of uranium 235 needed for a bomb. But his colleague Hahn immediately reminded him that during the war he had told him that only 50 kilograms were needed for a critical mass. After some prodding, Heisenberg admitted that, because it had appeared so unlikely that nuclear weapons could be produced during the war, he had not bothered to work out precisely how much fissionable material was needed.

Similarly, after newspaper accounts made clear that the Allies had also used plutonium in their atomic bombs, Heisenberg said that, because it had appeared so unlikely that they would be able to manufacture that element, they had not bothered to calculate fast-neutron reactions in plutonium. However, a few days later and having only their own research and the often misleading newspaper accounts to work with, Heisenberg presented a sophisticated and surprisingly accurate lecture on the Allied atomic bomb. So much for the theories that it was incompetence that had derailed the Nazi nuclear weapons project, or that the Germans had deliberately slowed down or diverted their research to deny atomic bombs to Hitler.

It is important to separate the question of whether the German scientists knew how to make atomic bombs from those of whether the Third Reich could have manufactured nuclear weapons before the end of the war, or of whether the scientists wanted to make atomic bombs for the National Socialists. The press reports of the attacks on Hiroshima and Nagasaki touched off a varied but pronounced reaction among the detainees. Several of them argued at first that they could not have made atomic bombs, not because of their own shortcomings, but because of the lack of vision, unwillingness, or the inability of the National Socialist leadership.

But the controversy surrounding the Farm Hall recordings has revolved around whether these scientists would in fact have made atomic bombs. The Op-

eration Epsilon transcripts demonstrate that von Weizsäcker did indeed try to convince his colleagues that they had not wanted to make nuclear weapons, but rather than these arguments constituting a simple cover-up, as many have alleged, they were arguably more of a concerted attempt to persuade himself and his colleagues to revise their own memories to put a better face on an increasingly problematic past. His colleagues were not forced to agree with von Weizsäcker, although in time many did. However, the transcripts also make clear that von Weizsäcker's arguments most probably inspired the postwar myth of a conspiracy against Hitler. Von Weizsäcker argued that history would record that the Americans and British had made an atomic bomb, but that the Germans had produced a workable and peaceful nuclear reactor under the Hitler regime.

None of these questions was as important to the scientists as their professional future in the postwar environment they foresaw: tension if not war between the United States and the Soviet Union; and strict control of science in Germany in general and of nuclear-fission research in particular. Several of the detainees were apprehensive about how they would be treated when they returned home and were deeply mistrustful of the Soviet Union. These fears were balanced in part by a hope that the work they had done on uranium would make their collaboration an attractive prospect to the western powers.

The mood of the detainees brightened considerably once it was clear that their release was imminent. Indeed, Heisenberg practically dictated where he wanted to return to, the University of Göttingen, one of the few intact universities in the American or British zones. The British occupation authorities subsequently made great efforts to ensure that he and his colleagues were as comfortable as possible in postwar Germany.

The Farm Hall transcripts are an interesting historical source, but tell us nothing new about the German atomic bomb. But their release does mean that no one can pin their hopes on new revelations or secret knowledge. □

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Language for a polyglot readership

What are the reasons why the English-language literature of science is understood only with difficulty elsewhere? And what might make it more accessible?

In June last year, this journal carried a photograph of a lion on its front cover to mark the publication of a Letter referring, among other things, to the determination of the status of a lion within the pride to which it belongs. It seemed natural, even smart, to add to the illustration of the lion the caption "Prides and prejudice": 'pride' is the collective noun for a group of lions, and *Pride and Prejudice* is the title of a novel by Jane Austen, arguably the second-best of all British women novelists. But more recently, a Japanese journalist complained to a colleague at the caption, explaining that his translation machine, essentially an English-Japanese dictionary, could make no sense of "Prides and prejudice".

A little thought should have shown us quickly why this caption would mean very little to a computer program designed to help Japanese to read English. The sobering thought is that the same caption may also have meant very little to anybody who happened not to know either the collective noun for lions nor the title of Jane Austen's novel, many of whom may shamefully have been English speakers, perhaps even Britons. Technically, the fault with the caption is that it is a play on words whose meaning may have been clear to a small section of *Nature's* readership, but mystifying to most.

Allusions are not in themselves impediments to understanding. When they are understood, they may shorten an argument or enrich its associations. But when they are not understood, they are worse than useless, but are puzzling, confusing and distracting. It is therefore of some importance to know where to draw the line between acceptable and unacceptable allusions.

Thus the sentence "A small step for DNA sequencing, a giant step for ReddyKwik Inc." will usually require no further parsing. ReddyKwik is telling its customers that it believes its latest new product to mark the beginning of a new era in its commercial history, and that it also wishes to invest the development with the sense of occasion attending Neil Armstrong's first walk on the Moon. But even that allusion may fall flat, for example in the former Soviet Union. Its effect will also fade as time passes.

Some allusions to classical history also 'travel', as people in the advertising industry sometimes say. 'Herculean' is, for example, an adjective in English and many other languages, although Augean is not, requiring readers to know that one of the tasks imposed on Hercules was to clean the Augean

stables. Biblical allusions must be used with care. Would 'Noah' feature in the Japanese journalist's translation machine? Why should the tale of Adam and Eve be better known in India than the tale of Lord Krishna is known in Western Europe? And it would be asking too much of readers' imagination to be told of a person who had changed his opinion of the beneficial effects of megadoses of vitamin C that "Palo Alto [the Californian town near which Linus Pauling lives] was his Damascus"; St Paul's conversion on the road to that city is a part of general knowledge in what is loosely called Christendom; elsewhere, it is not.

For a journal such as this, whose readers include perhaps 40 per cent for whom English is not their first language, allusive writing is a disservice and should usually be avoided. In any case, it would be unreasonably immodest to claim that allusive writing is commonplace in *Nature* or that it substantially enriches any group of readers' understanding.

This is one of the conclusions that might have been reached at a meeting of *Nature's* senior editorial staff a few days ago. It is part of an answer to one of several questions of editorial policy: how to make an international journal more accessible to a polyglot audience. The question is more easily asked than answered.

Not that it is difficult to specify other categories of usage that deserve as much caution as allusions. Slang, for example, which some would ban on aesthetic or even doctrinal grounds, is notoriously ill-travelled and impermanent. Cliches are in the same case; it is likely that only in Britain do politicians include the phrase "at the end of the day" (meaning 'ultimately', 'eventually' or 'in the end') in every other sentence; it is to be hoped that even they will soon let the practice lapse.

Unfortunately, these restrictions (desirable for other reasons) are not a solution to the general problem, whose roots lie elsewhere. And it is worth recalling that, not so long ago, an article in this journal demonstrated that even English-speaking readers would find the vocabulary of articles and letters in *Nature* substantially more difficult than that in some comparable and most generally circulating publications (*Nature* 356, 739-740; 1992).

That should not necessarily apply to the parts of this journal that are written or at least controlled by the editorial staff, but some features of scientific prose seem to be infectious, notably the habit of forming

portmanteau adjectives (such as 'DNA-dependent cytosol-controlled' as a qualifier of 'polymerase', if there is such a thing). English-speaking readers get used to these adjectives, but others have to unravel their meaning at every encounter.

But even simply structured words can make difficulties. *Nature* has tried over the years to contribute to a solution of the difficulty of vocabulary by choosing one of several alternative words with the same meaning. When 'while' and 'whilst' have the same meaning, will it not simplify a reader's understanding to settle for the first and to ban the second? To some degree, perhaps, but only to a marginal degree.

The underlying dilemma is that it is not possible to go too far in this direction without making prose sound like baby-talk. People in the trade are not put off by words such as 'asymptotic' (not to be confused with 'asymptomatic'), but it would serve no purpose for even non-English-speaking readers to translate them into simpler language. It is not in the vocabulary of science that the difficulty lies, but in the general vocabulary that holds it together.

Sentence structure is the other great impediment to understanding. The last sentence of the preceding paragraph, for example, begins with a passive clause whose effect is to dramatize the distinction between the two vocabularies, but which would probably have provided non-English-speaking readers with greater difficulty. Whether that is worthwhile is an open question.

These are interesting and important questions. *Nature* is anxious to improve its own performance and, in the process, to make the journal more accessible to its polyglot readership without diminishing such pleasure as its readers at present win from what it prints. There cannot be an absolute impediment to the improvement of the clarity of the text as perceived by people in different places. Probably the guiding principle is that if a text is clear in English (or, for that matter, in French), that it will also be more intelligible in Japanese.

Little is known about the practical impediments to polyglot understanding. *Nature* would be glad to hear from interested readers who believe they have something to contribute to the better understanding of the text in the front sections of the journal. And the remainder of the text? That is a different and more formidable problem which already engages many people's working attention. **John Maddox**

DNA recognition, warts and all

John Kuriyan and Stephen K. Burley

THROUGHOUT the life of a cell, its genetic blueprint is consulted and interpreted in response to various developmental and environmental signals. Central to this process is the recognition of specific DNA sequences by transcription factors, which then determine whether or not a particular gene is used. What are the mechanisms utilized by these DNA-binding proteins to enhance sequence-specific interactions? The answer is beginning to emerge from the analysis of the structures of protein-DNA complexes determined by X-ray crystallography and nuclear magnetic resonance.

On page 505 of this issue¹ Hegde *et al.* present the three-dimensional structure of the complex between a papillomavirus transcription factor and DNA, which reveals a new protein architecture as well as a new template for DNA-protein interactions. This DNA-binding fold and other previously determined structures (for reviews see refs 2 and 3) show that although the DNA comprising the genetic library is a conservative edifice, rich in information content but poor in structural variation, the proteins that serve as librarians are not predictably dull. Rather, transcription factors display all the architectural flamboyance characteristic of globular proteins.

Regions

The papillomaviruses are a family of DNA viruses that cause warts in humans and other mammals (for review see ref. 4). Proteins encoded by the viral gene E2 play central parts in the regulation of transcription and viral DNA replication, and are thought to consist of three functionally independent regions. The amino- and carboxy-terminal segments are highly conserved and constitute the activator and DNA-binding segments, respectively, while a central, non-conserved region appears to form a spacer between them. The 85-residue DNA-binding region will bind to specific DNA sites with high affinity, and is the subject of the X-ray structural work of Hegde *et al.*¹

Like many viral transcription factors, E2 protein binds to palindromic DNA sequences as a dimer, presumably as a means of maximizing specificity while minimizing the size of the E2 gene. It does not, however, resemble any known DNA-binding module, such as the helix-turn-helix, zinc-containing, leucine zipper or β -ribbon proteins^{2,3,5}. Rather it forms a dimeric eight-stranded antiparallel β -barrel structure (see Fig. 2, page 507) that presents, on the surface of the

barrel, two symmetrically disposed α -helices (the DNA-recognition helices). Although β -barrels are a common architectural feature in proteins (witness the number of enzymes that contain the β -barrel found in triose phosphate isomerase), E2 is the first example of a structure in which a barrel is formed by the dimerization of two polypeptide chains. This feature explains why the dimer is so exceptionally stable and cannot be dissociated, except under denaturing conditions. Each polypeptide chain contributes half of the hydrophobic core that forms the interior of the dimeric barrel. Two monomers therefore have a strong incentive to pair and exclude water, and the dimer is further stabilized by hydrogen bonds that form the seams of the barrel.

The two recognition helices are positioned such that both cannot make simultaneous contact with linear B-form DNA. Instead, the DNA bends more or less smoothly around the E2 protein into an arc that engages both α -helices within the major grooves (for comparison, this curvature is comparable to that of DNA spooled about the nucleosome core particle in chromatin). Deformations from ideal B-DNA character are now emerging as a common feature of protein-DNA interactions, but it remains to be seen whether sequence-dependent variation in the deformability of DNA is a general determinant of the specificity of DNA-protein interactions.

E2 interacts with 17 highly conserved target DNA sequences in the papillomavirus genome, and the crystal structure shows a network of interactions that seem well suited to confer specificity. All side chains that make direct contact with the target sequence are presented by the aptly named recognition helix. A characteristic of these interactions is that they are interwoven: a protein side chain makes contact with more than one base pair, and the identity-determining base pairs in turn interact with more than one side chain. These interlocking interactions are probably the basis for the high degree of conservation observed in both the target DNA sequence and the E2 protein.

Although the structures of protein-DNA complexes are beautiful to look at, attempts to codify the underlying interactions are decidedly problematic. The first high-resolution crystal structures of such complexes were reported four years ago, and the dozen or so structures we now have in hand suggest that, like protein folding, the problem of DNA recognition will not be easily

understood³. The elegant simplicity of information storage in DNA is not reflected in the structures of proteins that recognize DNA: nature's rule for designing these proteins seems to be that anything goes. The particular interactions between protein side chains and DNA base pairs depend in a highly complex way on the conformation of the entire assembly, and are not simply dictated by the DNA sequence alone.

Roles

Likewise, the particular roles played by protein and DNA structural elements differ from case to case. For example, E2 uses an α -helix to present side chains for interactions with DNA base pairs. In this case, the α -helix runs roughly parallel to the major groove. Other protein-DNA complexes show a range of orientations for the α -helix, and also show different parts of the helices providing contacts with DNA³. Another class of transcription factors uses pairs of β -strands that bind to the major groove of DNA⁵, while there are yet others, such as the TATA-box-binding protein (for review see ref. 6), that appear to make specific contacts with the minor groove.

The determination of the three-dimensional structure and the resulting tabulation of hydrogen bonds and other interactions is just the starting point for understanding the physical chemistry of DNA-binding specificity, which must take into account interactions with water molecules, counterions and with non-specific DNA sequences. Unravelling these details will keep theoreticians busy for a while yet.

In the meantime, we look forward to augmenting this rich harvest of DNA-binding modules with structural information about the equally important activation domains responsible for communication with other elements of the transcription apparatus. Such domains have not yet yielded to structural analysis, perhaps because they are less likely to be stable enough to make the application of crystallography and NMR straightforward. Ingenuity, as well as the application of other physical techniques, will be required to work out a complete picture of transcription factor action. □

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End of the 'Uniramia' taxon

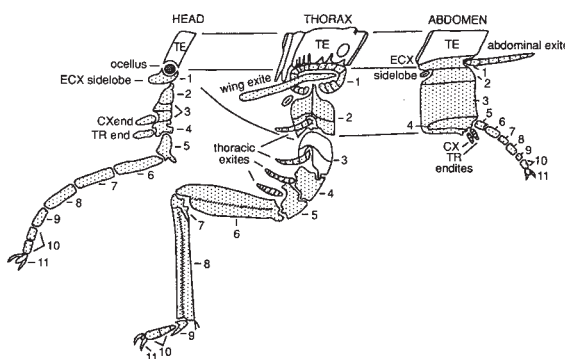
William A. Shear

ONE of the most extensively debated subjects in evolutionary biology has been the origin of the arthropods, that enormously successful group of animals which includes insects, spiders, crabs and their relatives, and a host of extinct types of almost every description. The focus of the debate was whether arthropods are monophyletic (derived from a common ancestor that was also an arthropod) or polyphyletic (derived from two or more different ancestors which were not arthropods). But now new interpretations of insect fossils published in the *Canadian Journal of Zoology* by Jarmila Kukalová-Peck¹ may at last settle the argument by driving the final nail into the coffin lid of the Phylum Uniramia, a concept central to the polyphyletic theory². If so, a major controversy in evolutionary biology has come full circle.

The monophyly of the Arthropoda was generally seen as secure, if not defined in exactly the terms used here, before the extensive work of the embryologists Tiegs³ and Anderson⁴, and Manton², perhaps this century's greatest comparative anatomist. Using evidence derived from developmental patterns and functional anatomy, 'Mantonians' argued that none of the major arthropod groups (trilobites, chelicerates, crustaceans and insects and their relatives) could be convincingly related to any other, and that all must have derived from separate annelid or annelid-like ancestors. In one form or another, this polyphyletic theory of arthropod origin enjoyed wide currency, appearing in general texts and evidently gaining considerable acceptance among invertebrate palaeontologists. The massive studies of comparative anatomy that buttressed this theory were extraordinarily intimidating to biologists whose interests and skills lay elsewhere.

The keystone of the polyphyletic theory² was the existence of the taxon called Uniramia. It was founded on the proposition that the insects and the four classes of arthropods often collectively referred to as myriapods were more closely related to a non-arthropod group, the Onychophora, or walking-worms, than to any other group of arthropods⁵. The last common ancestor of this group would have to be an onychophoran or annelid-like creature,

not an arthropod. Among numerous arguments advanced for the existence of the Uniramia as a taxon, two were most important. The first was the supposed homology of the claw-like jaw of the onychophorans with the mandible of the other uniramians. Manton maintained that both were formed from whole limbs, whereas the jaws of the other



Kukalová-Peck's ground plan¹ for the insect leg allows homologies to be established among all appendage-derived structures. In this drawing, the basic 11-segmented appendage, with both endites (inner branches) and exites (outer branches) in the articulations between the first five segments is modified differently on the head, thorax and abdomen. In the head, the basal appendage segment (ECX, epicoxa) is incorporated in the sidewall of the head and partially surrounds the ocellus. The next four segments (2-5, including the coxa (CX) and trochanter (TR)), form the body of the mouthpart (for example, the mandible) and the distal six segments appear as a palp, if one is present. On the thorax, the epicoxa surrounds and articulates the wing, which has developed from the epicoxal exite. The pleuron, or sidewall of the segment, has developed by the incorporation into the body wall of the second leg segment (2), so that the free leg now begins with the coxa (3). Exites on the thoracic legs can be clearly seen in well preserved insect fossils. In the abdomen, the epicoxa fuses to the dorsal body wall and the pleuron, or sidewall, is formed from the following four leg segments. The remaining leg segments project as abdominal leglets; the coxal and 'sternal' sacs and stylets seen on primitive insects and insect larvae are homologous to the coxal and trochanteral endites. The terminal genitalia of insects can also be homologized, structure by structure, to the basic 11-segmented leg and its several inner and outer branches.

arthropod groups were fundamentally different. These latter jaws were formed from limb bases, with the outer limb segments either strongly reduced or lost entirely. Secondly, the legs of uniramians, derived from onychophoran segmental lobes, were fundamentally one-branched, or uniramian.

To the modern evolutionary systematist, the philosophical flaw in the Manton argument² is its basis on differences and implied implausibility. Over and over again it is argued that arthropod groups are "too different" ever to have had a "plausible" common ancestor. Modern phylogenetic arguments, however, are

based on shared unique similarities, a contribution of the method of cladistics. To demonstrate that one section of a taxon must be removed and allowed to stand on its own with equivalent rank, it must be shown that it is more closely related to some group other than the members of its own former taxon. The relationship must be supported by more shared, evolved similarities (called synapomorphies) than exist with any other group. But Manton's theory failed to meet this structural requirement, and it cannot be restated in such a way as to preserve its main conclusions.

The homology of the mandible of onychophorans and uniramian arthropods has been thoroughly refuted by a number of lines of evidence. The appendage itself develops on a different segment⁶. Musculature clearly shows that the mandibles of crustaceans and uniramian arthropods are developed in both cases from a leg base, and are in fact homologous⁷.

Insect palaeontology now weighs in with new evidence. Kukalová-Peck, using data from decades of study of beautifully preserved insect fossils from Russia, shows that the mandible of primitive flying insects still retains the elements of the four basal leg segments, and that the outer ones have been lost¹. More importantly, she proposes a new ground plan for the arthropod leg (see figure), which includes fully eleven segments and allows the establishment of definitive leg homologies not only among all appendage-derived structures in insects (mouthparts, legs, wings, abdominal leglets, genitalia), but across the entire Phylum Arthropoda. She also demonstrates the presence on the legs of primitive insects of accessory outer branches, called exites. This means that the insect leg is only secondarily uniramous in living forms and that in the past

the legs of some 'uniramians' had a rich complement of branches. She proposes that both the uniramous leg and the biramous, or two-branched, leg (found in crustaceans, trilobites and chelicerates) are reductions from an ancestral polyramous leg. Thus follows the evolutionary and semantic doom of the formal term 'Uniramia'.

With the crumbling of this last part of the morphological evidence for the existence of Uniramia, we now return to a well-supported monophyly for Arthropoda. The use of the terms Uniramia and uniramian, both formally and informally, should cease. Most arthropod systemat-

RÉSUMÉ

Beastly yeast

An interdisciplinary team has taken a further step in tracking down the identity of *Pneumocystis carinii*, the mysterious organism which causes pneumonia in AIDS patients among others (*Molec. Microbiol.* **6**, 1903–1911; 1992). Once thought to be a protozoan, *P. carinii* was found to be a fungus only a few years ago. From comparative sequence analysis of part of the mitochondrial gene for the large subunit ribosomal RNA, A. E. Wakefield and colleagues now show that its closest allies are the ustomycetous red yeast fungi, the airborne spores of which are commonly found in all sorts of environments. There remains, however, the considerable challenge of growing the organism in culture, a prerequisite to further progress in unveiling the biology and epidemiology of its infection.

Burled prospects

THE hope that exotic forms of nuclear matter, termed nuclearites, exist fades with a report by 124 physicists working deep within the Gran Sasso mountain in Italy (S. Ahlen *et al.* *Phys. Rev. Lett.* **69**, 1860–1863; 1992). The authors, from the acronymically named MACRO collaboration are in fact looking for magnetic monopolies, another exotic species. But their underground cosmic-ray observatory should also see the hypothetical nuclearites, which are composed of up and down quarks, as is ordinary nuclear matter, and also strange quarks, normally seen only in high-energy collisions. The theory is that fragments of this 'strange-matter' could be stable and are flying around the Galaxy. But after sifting through 452,191 candidate detections, Ahlen *et al.* find none that fits the stiff criteria, even though the telescope should be sensitive to examples as light as 10^{-10} grams travelling as slow as 750 km s^{-1} .

Skeleton key

THE archetypal membrane skeleton is that of the mammalian red cell. It is responsible for the membrane's elasticity and durability. The microorganism *Euglena*, however, has a well-developed membrane-associated protein complex, which turns out to be quite different. Now J. A. Marrs and G. B. Bouck (*J. Cell Biol.* **118**, 1465–1475; 1992) have characterized the two constituent proteins called articulins. They are closely related, have molecular masses of 80,000 and 86,000 and are nothing like red cell spectrin or any other known protein. They have a core domain made of 12-residue repeats, rich in valine and proline. The predicted conformation is the β -sheet. The articulins are attached directly to the membrane by way of transmembrane proteins, do without actin, and form a new class of proteins for the collector.

ists seem to favour the name Atelocerata⁸ for a taxon including the Classes Hexapoda, Diplopoda, Chilopoda, Pauropoda and Symphyla, arthropod members of the ill-fated Uniramia.

And what of the Onychophora? New fossil discoveries⁹ suggest that a Phylum Lobopodia might be established in the near future to include the walking-worms, a few other isolated living groups

(Tardigrada, Pentastomida) and the growing number of fossil forms, including the formerly mysterious Burgess Shale *Hallucigenia*. The new phylum would be the closest relative of the (monophyletic) Arthropoda. □

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GAMMA-RAY BURSTS

Puritans and Cavaliers

Charles Bailyn

THE unexpected observation that astronomical γ -ray bursts are distributed isotropically across the sky has led to a dramatic shift in the fortunes of two competing ideologies relating to these mysterious phenomena. In particular, previously widespread support for the suggestion that these brief bursts of γ -rays come from neutron stars in our own Galaxy has retreated in favour of the idea that they originate at cosmological distances, which would naturally create the observed distribution. But the local neutron-star hypothesis may not be completely vanquished, as the paper by H. Li and C. D. Dermer in this issue (*Nature* **359**, 514–516; 1992) shows.

The difficulty posed by the isotropy result (obtained with the BATSE instrument on board the Compton Gamma-Ray Observatory: C. A. Meegan *et al.* *Nature* **355**, 143–145; 1992) for a galactic origin of γ -ray bursts stems from the fact that we are not at the centre of the Galaxy. From our vantage point, galactic objects are in general not distributed isotropically, but should be more prevalent in the direction of the Galactic Centre. The additional fact that there are fewer faint bursts than expected implies that we are seeing an 'edge' to the distribution of burst sources.

If the observed bursts are of galactic origin, their distribution should therefore exhibit a pronounced dipole moment, contrary to the observations. If, on the other hand, the burst sources are at cosmological distances, the observed isotropic distribution is to be expected, and the apparently bounded distribution would represent the edge of the observable Universe. The enormous distance of the sources would then require extraordinary intrinsic luminosities, one of several factors which led to the general

acceptance of a galactic origin of bursts in the first place.

The challenge for those still promoting a galactic origin for the bursts is therefore to discover a source population which is bounded, but nevertheless isotropic within the errors of the BATSE measurements. Because the spherical halo of our Galaxy extends out to more than ten times the distance from the Galactic Centre to the Sun, this challenge might still be met by a very extended galactic source population. However, galactic halo objects are usually concentrated towards the centre, and their distributions also exhibit a strong dipole moment. Thus if the burst sources are to be galactic, they must have a very unusual distribution, quite different from that of any known galactic population.

Both Li and Dermer and also D. Eichler and J. Silk (*Science* **257**, 937–942; 1992) suggest that the γ -ray bursters might be associated with newly discovered groups of high-velocity radio pulsars. Pulsars are young neutron stars with strong magnetic fields, and events on their surfaces have been considered prime candidates for the origin of γ -ray bursts for some time, although no generally accepted mechanism has been devised. They are generally thought to be produced by supernova explosions of massive stars, which occur only in the plane of our Galaxy. The supernova explosion gives the neutron star a kick, generally leaving the pulsar with a velocity of anything up to 200 km s^{-1} .

Recently, however, pulsars with much higher velocities have been discovered (P. A. Harrison, A. G. Lyne and B. Anderson *Mon. Not. R. astr. Soc.*; in the press). Li and Dermer consider the consequences of a population of pulsars with velocities greater than the escape vel-

ocity of the Galaxy, which is about 800 km s^{-1} . Such pulsars would speed away from the disk and never form a centrally concentrated equilibrium distribution. If the bursting behaviour does not begin immediately, but only after the pulsars have become radio quiet several million years after their birth, the neutron stars will be far from their birthplaces in a nearly isotropic distribution. Li and Dermer postulate that only these high-velocity pulsars create bursts, perhaps because of the exceptionally high magnetic fields which may be associated with them. Given these somewhat *ad hoc* assumptions, Li and Dermer show that the distribution of bursts from such objects could satisfy the current observational constraints. The distribution will not be perfectly isotropic, however, and the bursts which BATSE will see over the next few years should be enough to reveal the expected anisotropies.

Eichler and Silk start with the observation that some of the high-velocity pulsars are moving *towards* the galactic disk (an observation not addressed by Li and Dermer) and suggest that these pulsars may not after all be formed from the death of massive stars in the galactic plane. If instead they are formed by the coalescence of double-white-dwarf binary systems formed in the earliest stages of the collapse of the Galaxy, once again a nearly isotropic distribution might result. Of the various requirements imposed by this idea, one of the most stringent involves the masses of the required population of primordial stars. Because this stellar population is not currently observed, it must have contained very few stars significantly less massive than the Sun, as these slow-burning stars would still be shining today. On the other hand there must also have been few very massive stars which exploded as supernovae, as these would contaminate the collapsing galactic halo with heavy elements to a degree not seen. No known stellar population comes close to satisfying these criteria, although the extremely low metallicity of the gas out of which Eichler and Silk's proposed stars formed might have resulted in an unusual mass distribution.

While these two duos have been searching for a way around the isotropy problem, advocates of a cosmological origin of γ -ray bursts have been attempting to find mechanisms to account for the short duration, high energy and enormous implied luminosities of the bursts. Perhaps the most conservative such mechanism is discussed in a recent paper by R. Narayan, B. Paczynski and T. Piran (*Astrophys. J.* **395**, L83–L86; 1992). Once again neutron stars are implicated, but this time instead of being events on the stellar surfaces, the bursts are thought to result from mergers of

pairs of neutron stars or of a neutron star and a black hole. Certainly, double neutron-star binaries are known that will merge on astronomically short timescales, and the necessary energy would be released on short timescales in such an event. However, the radiative-transfer problems in determining the observable signature of such a cataclysm are far from resolved.

Clearly, those involved will continue to refine the various mechanisms and make more precise measurements of the

burst source distribution. In the meantime, it is a matter of taste whether one prefers to endure the after-the-fact pleading and resulting puritanical restrictions required by Li and Dermer and Eichler and Silk, or to revel in the cavalier outrageousness of the luminosities and phenomena required by the cosmological models. $\square$

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HIGH-PRESSURE PHYSICS

Make or break with fullerenes

Steve Duclos

As the structure with lowest internal energy, hexagonal graphite is the equilibrium structure of pure carbon at ambient conditions. Phase transitions between the many allotropes of carbon (cubic diamond, hexagonal diamond, hexagonal and rhombohedral graphite, and amorphous) at non-equilibrium conditions are of great practical and scientific interest. The fullerenes, van der Waals solids of the molecular fullerenes C_{60} , C_{70} and so on, recently joined the list of carbon allotropes, and as non-equilibrium structures provide another avenue for exploring carbon structural phase transitions and their kinetics.

Indeed, Moshary *et al.*¹ have now observed that solid C_{60} transforms at room temperature under extreme pressure to a novel form of amorphous carbon which is transparent at visible wavelengths and evidently very hard. This follows earlier reports of a transition from fullerite to cubic diamond under pressure² and of another from fullerite to hexagonal graphite under shock compression³. In addition to pure carbon allotropic transitions involving fullerite, there have also been recent advances in synthesizing a binary compound of carbon and nitrogen, with predicted properties rivalling that of cubic diamond.

Using absorption of visible light, Moshary *et al.* detect the expected pressure-induced change in electronic properties (a decrease of the band gap) in fullerite. But under approximately 25 gigapascals (250,000 atmospheres) of hydrostatic pressure a sluggish transition occurs which greatly increases the optical transparency of the sample at visible wavelengths. The new phase is metastable when quenched to atmospheric pressure at room temperature. In the Raman spectrum, peaks characteristic of C_{60} are no longer present in the quenched material, and are replaced by a broad feature indicative of an amor-

phous structure. Although the Raman spectrum of this collapsed phase of C_{60} is similar to that of known forms of amorphous carbon, the wavelength dependence of its optical absorption differs in structure and magnitude from previously observed amorphous carbons.

The authors also conclude that this new phase is quite hard, as it is able to support large pressure gradients within the apparatus. At 25 GPa, the nearly rigid C_{60} molecules have been forced together to the point where intermolecular bonding becomes important. The new phase results from nearly complete, and evidently random, C–C bond reconstruction following the destabilization of the molecule. The sluggishness and metastability are not surprising, as considerable energy is required to break the strong intramolecular bonds, and once broken these are not expected to reform. The hardness of the structure is probably attributable to a relatively high fraction of sp^3 -type (diamond-like) tetrahedral bonding in the carbon network.

Fullerite has also given more familiar forms of carbon when compressed under different states of stress. Regueiro *et al.*² find that when C_{60} is rapidly pressurized at room temperature in the presence of shear stresses, a rapid transition to cubic diamond occurs. Shock compression of C_{60} results in hexagonal graphite or amorphous carbon, depending on the pressure and temperature profile of the shock conditions. These transitions occur in the shock data of Yoo and Nellis³ at pressures as low as 17 GPa, and for Regueiro *et al.*², 20 GPa. The presence of shear stresses either accelerates the formation of intermolecular bonding or shears the molecules causing bond-angle changes and bond rehybridization, either of which can destabilize the molecule at a lower pressure.

Why these experiments have different results, and what drives C_{60} to the various allotropes of carbon, remains un-

clear. Sample purity, in terms of oxygen and hydrogen content, as well as fullerene species purity, could be important. Also, at ambient conditions the C_{60} molecules rotate freely, whereas at the pressures of these structural transitions the molecular orientations are frozen. Clearly the pressurization rate through the freezing transition, as well as the state of stress at that point, will determine the ultimate C_{60} orientation when the high-pressure allotropic transitions occur. How they freeze, either as an orientational glass or as an ordered structure, will probably influence how carbon bonds are reformed after destruction of the molecule.

Although the discovery of techniques to produce macroscopic quantities of fullerite has prompted this activity in the science of pure carbon, advances are also being made in the study of binary compounds of carbon. On the theoretical side, Liu and Cohen⁴ have used first-principles pseudopotential calculations to predict the stability of hexagonal C_3N_4 , the carbon analogue of β - Si_3N_4 . Using bond length and ionicity arguments they find that this structure should have a bulk modulus comparable to that of cubic diamond.

Experimentally it has proved to be a formidable task to incorporate significant quantities of nitrogen in a carbon lattice. But progress has been made by Chen *et al.*⁵ using reactive sputtering, another non-equilibrium growth technique. They have formed thin CN_x films that appear to be crystalline CN_x in an amorphous carbon matrix. Structural and bulk-modulus studies are needed to confirm that the crystalline form is the predicted C_3N_4 structure. But C-N bonding has been confirmed by infrared absorption; and nanoindentation, wear performance and friction studies reveal promising properties in the films.

The search for materials with properties such as hardness and thermal conductivity that rival those of cubic diamond has never strayed far from allotropes of carbon and compounds of its neighbouring elements boron and nitrogen. Important to this is a theoretical understanding of carbon bond destruction and reformation. As an allotrope of carbon, fullerite clearly provides a new tool to test these theories. □

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Every motion has its motor

Vladimir I. Gelfand and Jonathan M. Scholey

CHROMOSOME segregation, a dramatic event in the life of a cell, is coordinated by a piece of precision protein machinery, the spindle. This machine uses microtubule-based motor proteins, and four papers in this issue¹⁻⁴ tell us something of how spindle motors work and of how much we have yet to learn.

It was less than a decade ago that kinesin and cytoplasmic dynein were discovered as motor proteins capable of generating force for movement along microtubules. Subsequently, there has been a massive growth in the number of microtubule-based motor proteins that have been found to drive various types of intracellular movements⁵, including those associated with mitosis⁶. It is now clear that microtubule motors are required not only for chromosome move-

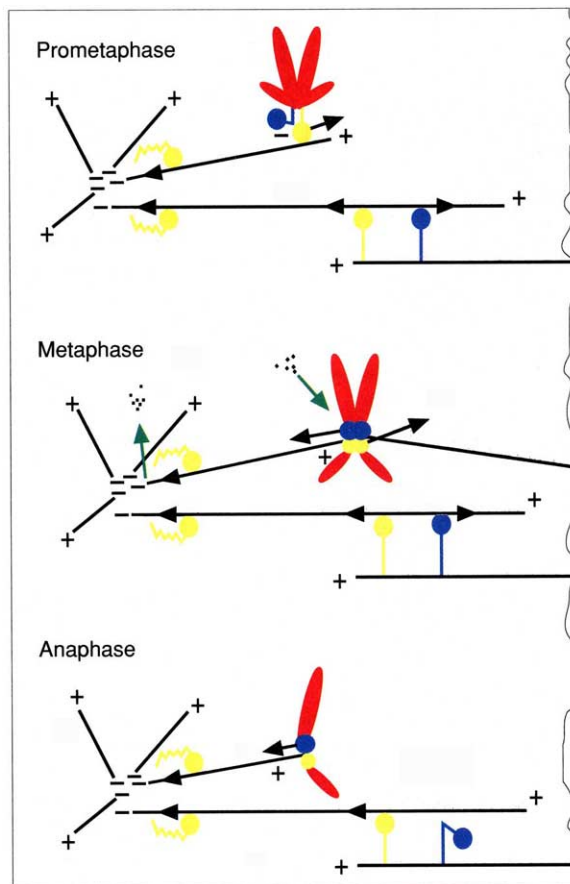
ment but even for the assembly and organization of the mitotic apparatus itself.

The mitotic spindle is a complex and dynamic, bipolar microtubule-based structure (see figure). It forms during prophase by interdigitation of microtubule arrays emanating from the two separating poles. The slow-growing (minus) ends of microtubules are always proximal to the pole. Some microtubules attach at their fast-growing (plus) ends to kinetochores of condensed chromosomes, which then oscillate back and forth during prometaphase as they congress to the spindle equator, where they lie at metaphase with their two kinetochores facing opposite poles.

Even at this apparently static stage, the spindle is very active; addition and

loss of tubulin subunits, at the equator and pole respectively, contribute to a steady-state microtubule flux. Moreover, counteracting forces seem to maintain the organization of the metaphase spindle, a balance of one set maintaining the equatorial position of chromosomes, another set determining the location of the spindle poles. During anaphase, the spindle undergoes a transition from its 'high energy' metaphase state as chromatids move slowly to the poles (anaphase A) and the poles are moved apart (anaphase B). The new papers describe motors that may be involved in different, possibly overlapping ways in the mitotic spindle. Three are members of the kinesin gene superfamily; the molecular nature of the fourth awaits identification.

One of the kinesin-like proteins, Eg5, participates in the morphogenesis of the metaphase spindle, which can be assayed *in vitro* using *Xenopus* egg extracts supplemented with sperm chromatin. As shown by Sawin *et al.*³, an antibody specific to Eg5 interferes with this process, so that the spindle poles are aberrant; instead of forming the usual bipolar structures, monoastral



Motor-dependent events of prometaphase, metaphase and anaphase. Only one of two half-spindles is shown. Lines represent microtubules with plus- and minus-ends marked. The forces acting on microtubules are shown by arrows directly on them, the forces acting on chromosomes (red) by arrows on motors near kinetochores, and subunit addition and loss by arrows in green. Plus-end motors, yellow; minus-end motors, blue. Eg5 is shown attached to a spindle matrix where it 'reels in' microtubules towards the pole. Motors that may possibly drive other movements detected here are discussed in the text. Inactive motors are shown unconnected to microtubules.

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2. Regueiro, M. N. *et al.* *Nature* **354**, 289 (1991).
3. Yoo, C. S. & Nellis, W. J. *Science* **254**, 1489-1491 (1991).
4. Liu, A. Y. & Cohen, M. L. *Phys. Rev. B* **41**, 10727-10734 (1990).
5. Chen, M. Y. *et al.* *Surface Coat. Technol.* (in the press).

structures with several poles in the middle result. Immunofluorescence microscopy reveals that on microtubules in normal spindles Eg5 seems to be more concentrated towards the pole. Moreover, in a motility assay, Eg5 functions as a plus-end motor, moving microtubules over glass with their minus ends leading at about $0.03 \mu\text{m s}^{-1}$ (that is with the same polarity as kinesin, but ten times slower). Sawin *et al.* propose that the Eg5 motor protein could attach to a matrix near the spindle pole, generating the forces that could contribute to spindle pole organization and generate poleward tubulin flux (see Fig. 4c of ref. 3). Interestingly enough, the proposed attachment of a plus-end directed microtubule motor to a spindle matrix was predicted by Pickett-Heaps⁷ from his cytological studies of mitosis, particularly in diatoms.

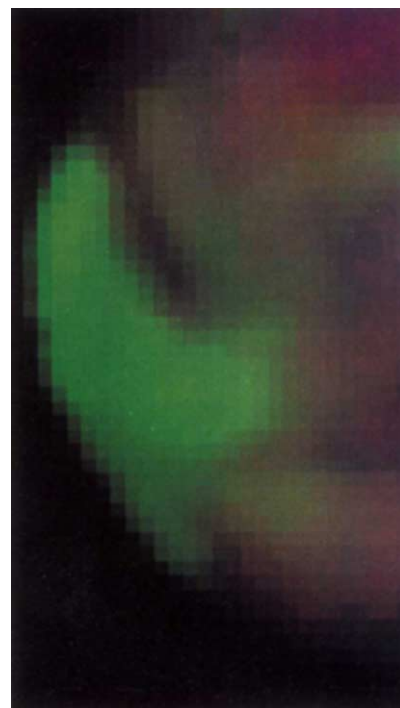
Before anaphase, Eg5 may help to maintain the organization of the assembled spindle by exerting pole-directed forces on antiparallel microtubules, as an opposing force pulls the poles together. Similarly, in the yeast *Saccharomyces cerevisiae*, Saunders and Hoyt have reported that the kinesin-like protein Kar3 may function as such a motor, pulling the spindle pole bodies together and thus counterbalancing the activity of yeast Eg5 analogues, encoded by the *KLPI* and *CIN8* genes (ref. 8; see also ref. 9).

Two of the other groups with papers in this issue^{2,4} used similar approaches to show that kinesin-like proteins play important roles in mitosis. In both cases monoclonal antibodies that interfere with normal spindle functions after microinjection into cultured cells^{10,11} were used to isolate cDNAs encoding polypeptides of the corresponding motor proteins. It turns out that both CENP-E (described by Yen *et al.*²) and MKLP1 (cloned by Nislow *et al.*⁴) are members of the kinesin superfamily with a characteristic motor domain (up to 500 amino acids long) attached to a coiled-coil α -helical extension — which, in the case of CENP-E, is enormous, almost four times longer than the stalk of conventional kinesin. Immunofluorescent microscopy showed that CENP-E is bound to kinetochores during prometaphase and metaphase, but at anaphase it translocates to associate with fibres of the spindle midzone. MKLP1, however, is never found on kinetochores but is restricted to the interzone of the anaphase spindle.

A remarkable feature of the work by Nislow *et al.*⁴ was the use of a modification of an *in vitro* motility assay to demonstrate that recombinant MKLP1 drives antiparallel microtubule-microtubule sliding. Geometrically, this movement *in vitro* is equivalent to the microtubule-microtubule sliding that underlies

Venus night lights

THE night sky on Venus is not so dark. In common with other planets, Venus is bathed in a pale glow of infrared light resulting from photochemical reactions in its upper atmosphere. On page 516 of this issue, D. Allen and colleagues present high-resolution images of the nightside airglow over Venus, which they use to track atmospheric circulation. The connection is that the airglow is the product of a giant photochemical reaction vessel encompassing the venusian mesosphere. Carbon dioxide on the Sunwards side of the planet (97 per cent of the atmosphere is CO₂) is heated and photolysed to generate oxygen atoms. These are carried by high-altitude winds to the nightside, where the cooled air descends. With increasing pressure, three-body reactions between oxygen atoms and additional species allow molecular oxygen to form, but in an electronically excited state. It is at this point that the airglow starts, as the molecules radiate their excess energy (green in the image). On its low-altitude return trip, the oxygen reacts with carbon monoxide to regenerate CO₂. By monitoring the airglow when Venus's nightside faced



towards us, Allen *et al.* found dramatic changes in the local wind conditions over periods as short as an hour — the beginnings of a meteorology for the planet's upper atmosphere. R.P.

anaphase spindle elongation¹², suggesting that MKLP1 may drive anaphase B. It is however possible that MKLP1 is involved earlier on in the formation of the metaphase spindle, because microinjection of the antibody CHO1 directed against MKLP1 leads to arrest of the cell cycle in prometaphase¹¹.

What regulates the association of these mitotic motors with spindle components? The motor protein CENP-E is degraded upon completion of mitosis and is not resynthesized until late in the next interphase; thus its presence in cells is restricted to M-phase, when it is needed. The predicted sequence of the MKLP1 polypeptide, on the other hand, contains a nuclear localization signal which seems to target the motor to the interphase nucleus, allowing contact with microtubules only during mitosis following breakdown of the nuclear envelope. Further details of regulation should also prove to be illuminating; what, for example, controls the striking translocation of CENP-E from kinetochores to fibres of the midzone during anaphase?

A comprehensive analysis of motor functions in the kinetochore will require the reconstruction of a functional complex from individual components. An important step in this direction has been taken by Hyman, Middleton and co-workers¹ who report that an isolated yeast protein complex that specifically binds to centromeric DNA¹³ contains the

motor activity. This activity moves latex beads with the attached centromeric DNA along microtubules towards their minus ends, at speeds close to the rate of anaphase chromosome movement in animal cells. The specificity of the isolation procedure was beautifully demonstrated by showing that the motor complex does not move the beads containing mutant centromeric DNA. The identity of the polypeptide(s) within this complex responsible for the motion is not yet known, but the combination of biochemistry and *in vitro* motility assays, and the powerful genetics of *S. cerevisiae*, promises rapid progress.

We can now see that motor proteins,

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of which there may be many more, play subtler roles in intracellular organization than was once imagined. Some microtubule motors involved in mitosis and other intracellular processes do not simply move components from one place to another in the cell, but may help maintain the dynamic steady-state organization of cells. For example, we think that one set of motors may generate the opposing forces that hold the chromosomes on the equator of the metaphase spindle while another positions the spindle poles prior to anaphase. Similarly, microtubule motor proteins in interphase cells may burn ATP to generate an 'isometric' tension that maintains intermediate filaments and intracellular

membrane tubules in a dispersed state^{14,15}. These results may surprise everybody except muscle physiologists who study molluscan catch muscles — these muscles use the actin-based motor protein, myosin, to contract and maintain resistance to stretch for hours, days and even weeks. □

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EMBRYOLOGY

The quest for immortality

Anne McLaren

GERM cells — the cells whose descendants, if they survive at all, end up as sperm or eggs — are in a sense all potentially immortal. Yet those of us who have tried to culture mammalian germ cells have been tantalized by our failure to persuade them to survive and proliferate *in vitro* for more than a week or two at the most^{1,2}. On a feeder layer they do better, but not much better³. Some growth factors have been tried, but with only marginal effects on survival time^{4,5}. It seemed possible that germ cells had a finite proliferative capacity *in vitro*, as indeed they do *in vivo*, before

entering gametogenesis.

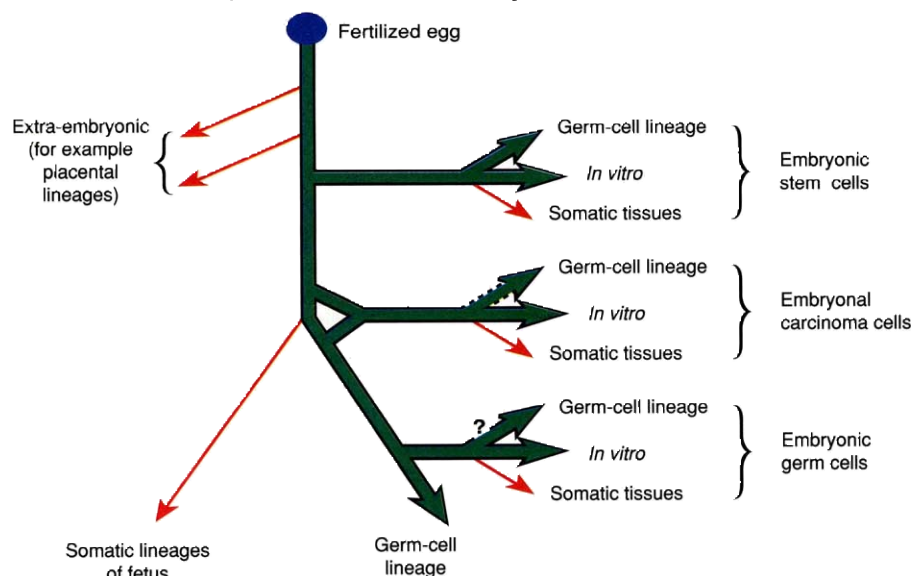
But where single factors failed, a blend seems to do the trick. On page 550 of this issue⁶, a paper from Peter Donovan's laboratory (Resnick *et al.*) reports the long-term survival and proliferation of mouse germ cells in culture. Three growth factors were required: basic fibroblast growth factor (bFGF) and soluble leukaemia inhibitory factor (LIF) were added to the medium, while the feeder layer expressed the transmembrane form of steel factor (SLF).

Under appropriate culture conditions, explanted skin cells continue to look and

behave like skin cells, and nerve cells continue to look and behave like nerve cells. But the cultured germ cells of Resnick *et al.* neither look nor behave like germ cells. Initially motile and polarized, after a few days these EG (embryonic germ) cells form large multi-layered, densely packed colonies of cells resembling the ES (embryonic stem) cells that can be derived by explanting preimplantation embryos, or the EC (embryonal carcinoma) cells that form the pluripotent stem cells of embryonic tumours. Transplanted under the kidney capsule, or allowed to attach to a plastic culture dish, ES and EC cells give rise to many differentiated cell types; injected into a blastocyst both will colonize all somatic tissues of the resulting chimaera. Only one case of germ-line chimaerism has been claimed using EC cells⁷, but ES germ-line chimaeras must by now number many hundred, and constitute one of the most popular routes for making transgenic mice.

How about EG cells? A group led by Brigid Hogan (Matsui *et al.*⁸) has also reported long-term culture of mouse germ cells. When bFGF and LIF were added to a feeder layer of cells expressing the transmembrane form of SLF, mouse germ cells could be maintained in culture for at least 20 passages. Again they formed colonies of densely packed cells, described as resembling ES cells in morphology. Transplanted into nude mice, or allowed to attach to a plastic surface in culture, they gave rise to many differentiated cell types. Most telling, Matsui *et al.* have injected their cells into blastocysts and produced four chimaeric mice. Only two contained a substantial proportion of donor cells: of these, one died before weaning and the other proved to be sterile. The question of whether EG cells can colonize the germ line therefore remains open.

What is the relationship between ES, EC and EG cells? This raises the whole question of the nature and origin of ES and EC cells (see figure). Embryonic stem cells are derived from the inner cell mass component of blastocysts that are developing *in vitro*: they have been compared to 4- or 5-day post coitum primitive ectoderm cells, though their protein synthetic profile on two-dimensional gels does not exactly equate with either. Embryonal carcinoma cells are recovered from teratocarcinomas formed when 6-7-day post coitum embryos are transplanted beneath the kidney capsule: there is circumstantial evidence that might indicate that they were derived only from the primordial germ cell component of the transplanted embryos⁹. If so, they would be the exact equivalent of EG cells. One might, therefore, predict that EG cells, like EC cells, would rarely if ever colonize the germ line.



In the intact embryo, the potentially immortal primordial germ cells are formed during gastrulation, while the rest of the embryo forms mortal somatic tissue. The ES, EC and EG cells are derived from the embryo at different points during development. All will survive and proliferate for long periods in culture, and can readily be induced to differentiate into somatic tissues, but only ES cells have so far been shown to colonize the germ line at a reasonably high frequency. Mortal lineages are shown with thin red arrows, potentially immortal lineages with thick green arrows.

The similarity between EC and EG cells is strengthened by the observation that early male genital ridges from a very few mouse inbred strains may give rise to EC-cell-containing teratocarcinomas if transplanted under the kidney capsule¹⁰ (but these EC cells have never been claimed to colonize the germ line), whereas germ cells from early male genital ridges will differentiate into EG cells in bFGF/LIF/SLF-supported culture (but Matsui *et al.* have not yet tested the differentiative capacity of these EG cells).

In the embryo, primordial germ cells can first be identified as a group of some 50–100 cells (probably not just the eight more differentiated cells highlighted by Resnick *et al.*) tightly clustered in an extra-embryonic location during gastrulation¹¹. They are believed to derive from undifferentiated cells of the primitive ectoderm that have moved (or been moved) to this position at the onset of gastrulation, when the primitive streak first appears, that is, during the period that EC cells can be derived from transplanted embryos. Their extra-embryonic sequestration is postulated¹² to be important for the maintenance of pluripotency, protecting them from those influences that in somatic cells lead eventually to terminal differentiation and death. It seems paradoxical that pluripotent EC cells supposedly derived from germ cells will colonize the somatic lineages readily but the germ cell lineage rarely, if ever. Primordial germ cells are not undifferentiated cells. They have already begun to differentiate and may have lost some developmental potency; eggs and sperm are among the most highly differentiated cells in the body, and part of this differentiation must involve the reinstatement of full potency if subsequent embryonic development is to be achieved. Herein lies the central mystery of germ cell development.

So what of EG cells? If they turn out to be EC cells in another guise and fail to colonize the germ line, they will be of limited interest, though they could yield valuable information on the origin of genomic imprinting. Of greater value would be immortalized germ cells that remained as differentiated germ cells *in*

vitro. But perhaps EG cells have de-differentiated into something closer to ES cells — de-differentiated not necessarily in the sense that their developmental pathway has been traversed in the reverse direction, but merely that a previous state has been regained.

In that case, EG cells might colonize the germ line at least as readily as ES cells, thus opening up a new route for transgenic technology. Gene replacement or modification by homologous recombination requires ES cells, but so

far only mice (and possibly hamsters) have yielded ES cells. Agricultural and medical research would benefit greatly if these techniques could be extended to cows, sheep, pigs and rats: perhaps EG cells will show the way. Perhaps too they will teach us something about the molecular basis of developmental potency. □

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GEOMORPHOLOGY

Characteristic size of relief

Douglas W. Burbank

NEW and rapidly evolving approaches to the analysis of landscapes promise to change the way in which landscape characteristics (elevation, relief, slope, drainage density and so on) are integrated into multidisciplinary investigations of the Earth's surface. At a conference* on *Tectonics and Topography* last month, geomorphologists, geophysicists, structural geologists and others gathered to discuss the interrelationships and feedbacks between tectonic processes and the evolution of landscape. It became obvious that the ability to define landscape characteristics in a quantitative sense across broad areas could provide fresh insights and provoke questions that have not been previously addressed.

Digitized elevation data, whereby elevation estimates are collected and stored as a grid of points, are providing this new potential. Although individual digital elevation models (DEMs) have been used in the past, only recently have DEMs offering coverage of large areas of the Earth's surface become more available for basic research. Not only do these permit a digital representation of the landscape, but, through simple computations, it is now possible to quantify rapidly the topographic characteristics of areas ranging from local drainage basins to entire continents. The area–altitude distribution of land (hypsometry) is readily determined, and with an appropriate algorithm, mean slope can be estimated. Along a swath of specified width, the maximum, minimum and mean elevations and the topographic relief are easily

calculated. Although interest in and assessment of these characteristics is not new, and neither are some of the observations unexpected, the ability to determine them quickly, in a uniform format and across vast regions provides a precise, previously unattainable, large-scale overview.

Under Bryan Isacks's direction at Cornell University, E. Fielding, C. Duncan

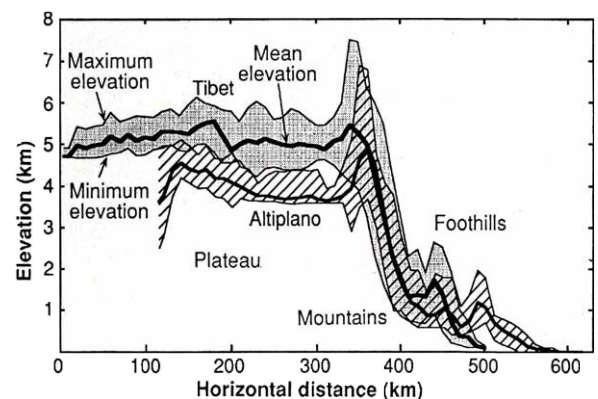


FIG. 1 Digital topographic data along 100-km-wide transects oriented perpendicular to the Himalayas and Andes.

and T. Gubbels have been analysing DEMs from the Andes and the Himalayas and developing techniques for derivation and display of the data. The results so far have been striking. Transects from the Tibetan Plateau, across the Himalayas and into the Ganges foreland (Fig. 1) show that, even within the area containing the highest summits in the world, the Himalayas have a mean elevation (5–5.5 km) that is essentially no more than that of Tibet. Given the contrasting local tectonic environments of these regions (active extension within Tibet and active contraction in the Himalayas), their comparable mean heights may reflect physical limits on the maximum sustainable elevation to which masses can be lifted above sea level (P. Molnar & H. Lyon-Caen *Geol. Soc.*

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*American Geophysical Union Chapman Conference *Tectonics and Topography*, Snowbird, Utah, 30 August – 4 September 1992.

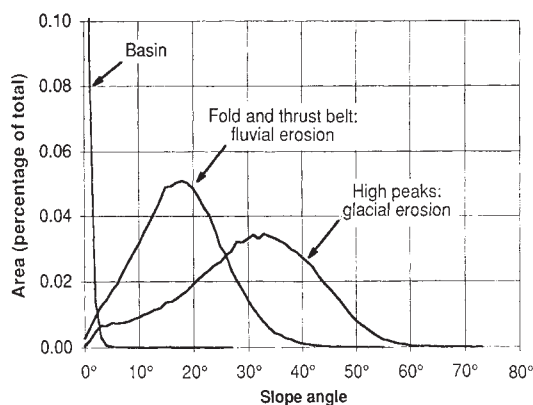


FIG. 2 Distribution of slope angles as a function of position within the Himalayan orogen.

Am. Spec. Pap. 218, 179–207; 1988). In contrast, topographic relief attains its highest values in the vicinity of the most lofty summits (a not unexpected result), but is nearly equal within the Himalayan foothills and the Tibetan Plateau. This type of topographic analysis has a wide appeal, because it yields data on mean conditions, which are most significant for modellers and geophysicists, as well as on deviations from the mean, quantities of key interest to geomorphologists.

Analogous transects from the Altiplano eastwards to the Andean lowlands reveal similar consistency in the mean topography across the mountains and the adjacent plateau (Fig. 1). Moreover, there is a remarkable coincidence in the slope (5°) of the mean topography from the core of the mountains to the plains in both the Himalayas and Andes, where the average elevation drops from around 5 km to 1.5 km across a span of 40 km. At a more detailed scale, Isacks's group estimate local slopes by fitting planes to 400-by-400-m windows of data. Although reliance on data points spaced 100 m apart leads them to underestimate local relief, it nonetheless serves to provide an array of slope estimates across broad areas. When placed in a regional context, dramatic contrasts appear between the slope distribution for the regions of the foothill fold-and-thrust belts and those for the higher peaks, where slopes are about twice as steep (Fig. 2). Within these two domains, the majority of foothill slopes fall between 15–20° and occur where monsoonal intensity is highest, whereas in the higher mountains, average slopes exceed 30° and are associated with glaciated landscapes.

Isacks proposed that there are two "erosional buzzsaws": one associated with fluvial erosion in the foothills and the other with higher-altitude glaciation. If true, the repeated lowering of snow-line in past glaciations might be invoked to explain the constant mean topography between the plateau and adjacent mountains. The efficacy of glaciers as erosional agents, however, is hotly debated:

some people argue that they actually protect slopes from significant erosion. Clearly, glacial erosion changes the shape of valleys, but does it excavate them more rapidly than do rivers? Few data are available to address this.

Given that the Himalayan mountain building has been sustained for millions of years, it might be anticipated that a steady-state topography prevails, such that slope angles over the long term are stable and are controlled solely by local rock types acted upon by local surficial processes. It is possible,

however, that elevated rates of erosion driven by glaciation and periglacial weathering have perturbed the equilibrium that commonly exists in areas where fluvial processes dominate. In its place, glaciers, large landslides and outburst floods may have moulded a landscape characterized by supercritical slopes and nonequilibrium topography.

Steeper slopes and deeper valleys, unloading the underlying mantle, could initiate isostatic uplift, as hypothesized by P. Molnar and P. England (*Nature* 346, 29–34; 1990). Once again, DEMs may allow one to assess this hypothesis. Although no experimentally based models for glacial erosion or denudation by landsliding are presently available, volumes of eroded rocks within the mountains can be readily calculated using DEMs if prior topography is known or assumed. Restoring these eroded rock masses back into the mountains, as done by J. Masek (Cornell University) for the Himalayas, permits prediction of the isostatic deflection their removal would have caused. In a similar fashion, a natural experiment in Papua New Guinea — where a pristine early Pleistocene surface has been domed up to 4 km above sea level and partially dissected — has been used to assess the isostatic impact of erosion and to demonstrate that erosionally driven uplift here is relatively unimportant in comparison with tectonically driven uplift (L. Abbott and J. Galewski, University of California at Santa Cruz).

From the fruits of these initial studies, it is clear Earth scientists will have no trouble digesting whatever topographic data are sent their way. Part of the question now is persuading the various agencies compiling the databases (the US Department of Defense has the most extensive and detailed) to make them as widely available as possible. □

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DAEDALUS

Active rigidity

Most engineering structures are limited not by lack of strength but by lack of stiffness. They fail not by breaking, but by bending or buckling. Some cars have 'active suspension' systems which sense and react against shocks and deformations. Daedalus now plans to extend this philosophy to hitherto passive engineering structures.

Imagine, he says, a thin hollow tube with a powerful laser beam shining straight up through the middle, without touching the sides. Suppose the tube begins to buckle out of true. The straight beam within it will graze the inside of the tube at the elbow of the bend. The material at that point will heat up and expand; the resulting restoring force will straighten the tube. In effect, the laser beam continuously scrutinizes the whole length of the tube, and counteracts perfectly every attempt at bending or buckling. The tube thus becomes infinitely rigid. It stands as straight and unbending as a beam of light.

The same effect might be achieved more neatly by evacuating the tube and firing an electron beam through it. Modern electron-optics can generate beams intense enough for welding, and highly parallel; furthermore, an anode at the far end of the tube could collect the beam as an electric current and recover its energy for recycling to the power supply. Either way, the massive conventional pillars, struts and girders of conventional engineering could be replaced by safe, narrow, actively rigid tubes. Elevated motorways could stalk the landscape on absurd-looking spindly legs; tower cranes could become as insubstantial as their cables, and street lamps could stand erect purely on their power leads.

More cunning still, actively rigid components could be reconfigured under central control. On an aircraft, for example, actively rigid wings would not only be far lighter than conventional ones, and far more rigid under changing aerodynamic loads; they could act as control surfaces too. Simply by shifting the aim of their internal lasers or bending their electron beams by a set of deflector coils, they could be precisely flexed or deformed to vary their lift and drag. In the same way, actively rigid swing bridges could open and close on demand, and actively rigid buildings under inertially-guided computer control could stand unmoved by the most violent earthquakes.

The downside of this elegant principle is that any actively rigid structure will collapse instantly if the power is shut off, even for a moment. A fast-reacting self-contained back-up power supply will be essential. On the other hand, scrapping or demolition will be simplicity itself.

David Jones

Bubbles in volcanic systems

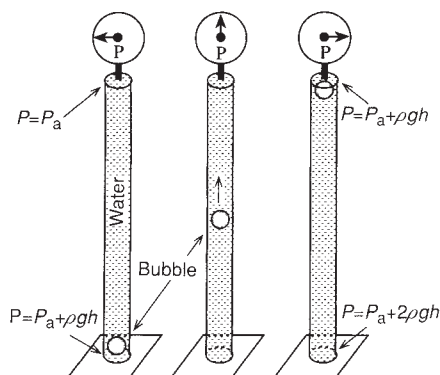
SIR — Many volcanic eruptions are triggered by processes occurring in magma reservoirs at a few kilometres depth in the crust beneath volcanoes. Although the approximate sizes and shapes of magma chambers and vent systems can be determined by geophysical measurements, their internal processes cannot be observed directly. At shallow depths magma contains gas bubbles, and we show here how the rise of bubbles in a magmatic system may lead to a pressure increase sufficient to fracture the surrounding rocks and cause an eruption.

For simplicity, consider a model system containing a single bubble initially at the base of a sealed reservoir of constant volume.

$P_{\text{fl}} = P_a$ at $h=0$ (top of the reservoir); and $P_{\text{fl}} = P_{\text{gas}} = \mu RT/V = P_a + \rho gH$ at $h=H$ (bottom of the reservoir), where P_{fl} is pressure in the fluid, P_a is atmospheric pressure, P_{gas} is gas pressure in the bubble, μ is molar content of the gas, and R is the gas constant.

The bubble can rise to the top of the reservoir due to its buoyancy or be carried by rising melt, but by the conditions listed above, its volume and pressure cannot change. When the bubble reaches the top of the reservoir, the pressures are: $P_{\text{fl}} = P_{\text{gas}} = \mu RT/V = P_a + \rho gH$ at $h=0$ (top of the reservoir); and $P_{\text{fl}} = P_{\text{top}} + \rho gH = P_a + 2\rho gH$ at $h=H$ (bottom of the reservoir). Thus, the bubble has transported the hydrostatic pressure at the base of the reservoir to the top, thereby increasing the pressure throughout the system by ρgH . We define this increase in pressure as 'advective overpressure'.

This concept has been met with some surprise within the volcanological community, so we constructed a simple experiment to demonstrate the effect. We used Plexiglass tube (2.4 m long and 5 cm in diameter) oriented vertically and filled with water (see figure) that was allowed to degas before the experiment. A 4-cm diameter air bubble contained in a simple red balloon was placed at the



Volume of the system is constant.

base of the water column, all other air was removed from the system, and the system was sealed. The balloon, which prevented diffusive gas exchange, was held at the base of the apparatus by an arm which, when released, allowed the bubble to rise. A fluid pressure gauge mounted at the top of the system indicated an increase in pressure from 1 to 1.25 bars as the bubble rose compared with a theoretical value of 1.24 bars.

Now consider a magma chamber of arbitrary width and with a height of 1 km. If a bubble were to rise from the base of the chamber to the top, the difference in hydrostatic pressure carried by the bubble to the top of the chamber would be $\rho g\Delta H \approx 800$ bars. A real magma chamber differs from our simple model in that magma is slightly more compressible than water, and the gas volume fraction (many small bubbles) of the natural system is less, reducing the magnitude of the pressure increase. Further, a vent would probably be opened long before overpressure reached 800 bars, but this example illustrates the potential potency of the mechanism of

advective overpressure.

In practice, the necessary conditions are probably only partially met in a magma chamber¹, as it is known that volcanoes deform before and after an eruption², and leakage can occur leading to opening of the system³. We can therefore consider the overpressure in a natural system as the balance between bubble rise rate and gas escape, as well as deformation of the edifice and compressibility of the magma. The mechanism of advective bubble overpressure is expected to supplement other sources of magmatic overpressure such as crystallization and magma recharge^{1,4,5}, but acts independently.

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A. A. Proussevitch

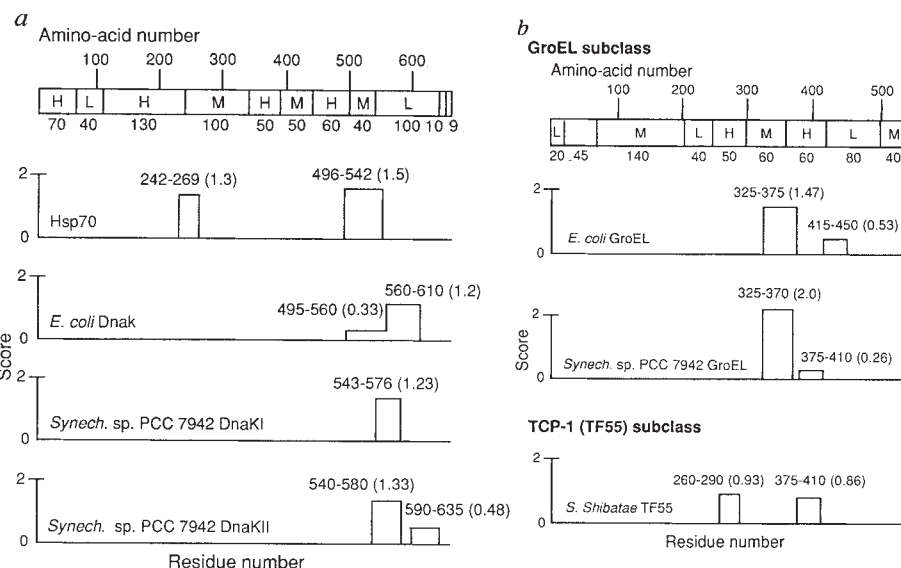
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Chaperones classified

SIR — J. Ellis in his News and Views article¹ arranges molecular chaperones into two groups, the hsp70 (DnaK) group and the chaperonin group, consisting of the GroEL (hsp60) subclass and

the TCP-1 (TF55) subclass. This division is based on phylogenetics and cellular localization. Molecular chaperones act sequentially to assist protein folding, and are involved in protein targeting and



Analysis of molecular chaperone structure using the Coils algorithm². a, Hsp70 (DnaK) group; b, chaperonins. Above the output from the algorithm are bars representing levels of amino-acid identity along the lengths of these proteins (adapted from ref. 6) (H, high; M, moderate; L, low; blank, none). Positions of predicted coiled-coil domains are denoted by amino-acid residue number and relative score quality. Sources of sequences: Hsp70, consensus sequence from ref. 6; *E. coli* DnaK, accession number GenBank K01298; *Synech. sp. 7942* DnaK and DnaKII, from *Synechococcus sp. PCC 7942* (R.W. and L.A.S., unpublished data); *E. coli* GroEL, accession number GenBank S01432; *Synech. sp. 7942* GroEL (ref. 3), accession number GenBank M58751; TF55, ref. 5.

complex assembly. Members of each group or subclass have very similar amino-acid sequences when compared with members of the same group or subclass, but there appears to be little sequence similarity between groups. We believe that this grouping of the molecular chaperones is further reinforced by analysis of structural motifs. Members of the hsp70 group and the GroEL subclass each have in common regions of structural similarity that may form coiled-coil domains, whereas this type of structure appears to be missing among the TCP-1 proteins.

Lupas *et al.*² first noted that the hsp70 group possesses regions likely to form amphipathic helices similar to those found in proteins that form coiled coils. They designed a computer algorithm which awards high scores to amphipathic helices containing patterns of hydrophilic and hydrophobic amino acids arranged in heptad repeats within a moving window of 28 residues. This algorithm identified more than 200 proteins when tested against GenBank, including the myosins, laminins and the leucine zipper regions of transcriptional regulators. Proteins known not to contain coiled coils, such as globins and immunoglobulins, were never identified by this algorithm.

We applied this algorithm to other members of the hsp70 group with similar results, including two copies of DnaK from the cyanobacterium *Synechococcus* sp. strain PCC 7942 (*Synech.* sp. PCC 7942) which we have recently sequenced. We extended this analysis to the GroEL subclass (ref. 3) as shown in the figure. Again, members of this subclass are predicted to possess coiled-coil domains. This motif is also shared by highly divergent members of this subclass such as the plastid chaperonin-60 α - and β -polypeptides⁴ (GenBank accession numbers M35599 and M35600, data not shown). The coiled-coil domains in all cases are predicted in regions of these proteins which show moderate to low similarity between amino-acid sequences in members of each group or subclass.

By contrast, TCP-1 sequences from mouse and *Drosophila* are not predicted to form this type of structure (GenBank accession numbers P11983 and P12613, data not shown). Analysis of the TF55 member of this subclass revealed two such possible structures each with marginal score quality.

This type of structural analysis further

supports the division of the molecular chaperones proposed by Ellis and raises interesting questions about the possible function of coiled-coil domains within two of the three types. We suggest two possibilities for these domains: (1) involvement in the binding of nascent polypeptides; or (2) involvement in the oligomerization of these two types of molecular chaperone.

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Checkpoint policing by p53

SIR — A recent News and Views¹ proffers a seductive model in which the tumour-suppressor protein p53 is integral to a checkpoint that is responsive to DNA damage. Although this model addresses some of the conundrums in our current understanding of p53 action, it leads to a few simple predictions that are not supported by evidence from this or other laboratories.

It is not clear whether the proposal is for p53 to control a G1 checkpoint (thus preventing progress through the 'Start' restriction point) or an S-phase checkpoint, which would allow cells to arrest DNA synthesis when the DNA becomes damaged. We assume that it is the latter as it is implicit in the model that the checkpoint closes down DNA synthesis. The effects of damage to DNA on its replication are complex and depend on the nature of the damaging agent (for example, see ref. 2). For the sake of simplicity we will confine our comments to damage from ionizing radiation. We have previously shown that DNA synthesis is comparably inhibited by ionizing radiation in primary fibroblasts and in fibroblasts transformed by simian virus 40 (SV40)³. (The model assumes that loss of p53 function by binding to SV40 T antigen is equivalent to mutational loss of p53.) Further experiments using SV40-transformed cell lines showed that this inhibition was mediated by a *trans*-acting factor^{4,5}.

Irrespective of whether p53 mediates a G1 or S-phase damage-responsive checkpoint, the proposed model would predict that cycling SV40-transformed cells should, when compared with primary cultures, show increased sensitivity to killing by ionizing radiation. But it has been shown that a series of SV40-transformed human fibroblast lines are, in fact, more resistant to ionizing radiation than the corresponding primary cell strains⁶. When damaged by other agents

like ultraviolet radiation, the sensitivity of primary and SV40-transformed lines is very similar. The sensitivity of many tumour and SV40-transformed cell lines to alkylating agents is known to be caused by specific loss of the O6-methylguanine DNA methyltransferase repair enzyme. Thus, although some tumour cell lines may be sensitive to some types of DNA damage, there is no evidence that tumour cells (or SV40-transformed lines) in general are more sensitive to DNA damaging agents than normal cells. We conclude that, although the concept of p53 as a 'molecular policeman' responding to DNA damage is neat and provocative, the wrong suspect may have been apprehended.

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LANE REPLIES — Carr *et al.* argue that I have apprehended the wrong suspect as a 'molecular policeman' because they know of other factors that control sensitivity to alkylating agents, and because they do not find differences in the sensitivity of primary and SV40-transformed cells to ionizing radiation. I have no argument with their first point, and, of course, it is clear from the large number of different mutations that confer sensitivity to genotoxic agents in man and yeast that p53 is not the only factor that 'guards our genome'.

I do, however, maintain the view that inactivation of the p53 pathway is a critical rate-limiting step in the development of neoplasia and that it functions through a checkpoint mechanism. A recent study by Kuerbitz *et al.*¹, extending their earlier work², shows clearly that p53 is responsible for a G1 checkpoint control that operates after exposure to low doses of ionizing radiation. These authors demonstrate that the checkpoint is absent in cells that express either no p53 or mutant p53. Critically, they can restore the control by introducing the wildtype p53 gene back into the null cells. The response certainly occurs in physiologically relevant circumstances, as we have recently shown that a dramatic induction of p53 occurs in the basal layer of human skin after mild exposure to solar mimetic ultraviolet radiation³.

NATURE · VOL 359 · 8 OCTOBER 1992

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Why then do Lehmann and his colleagues not see any difference in the response of their primary and SV40-transformed cells to ionizing radiation? One possibility is that the p53 pathway is not functioning correctly in their primary cells. Several studies now indicate a very strong selection against p53 function during the establishment of cells in culture (for example, ref. 4) and it is possible to overcome the response to p53 through the activity of other genes^{5,6}. The difference in genetic stability between established cells and primary cells is, for example, well documented by the high frequency of gene amplification events found only in cell lines (for example, ref. 7). Alternatively, the problem may be methodological — as Kuerbitz *et al.* discuss, G2 checkpoint controls still

operate in their p53-deficient cells and care is needed to distinguish this from the G1 arrest¹. Not all policeman are equal, and some are more easily evaded than others!

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Swallowing ornamental asymmetry

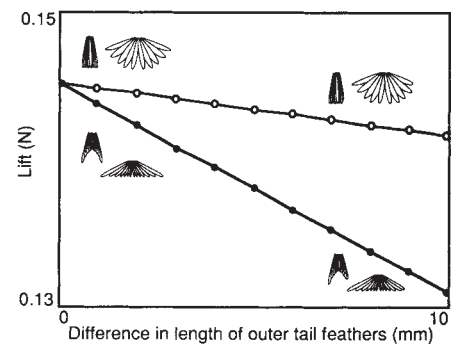
SIR — Møller¹ provides convincing evidence that the reproductive success of male swallows (*Hirundo rustica*) is independently increased by both the length and degree of symmetry of their outermost tail feathers. He suggests that the greater success of males with symmetrical tails arises not through an enhanced ability of males to provision their young or win intrasexual contests over females, but through female preferences for symmetrical as well as large ornaments. Because fluctuating asymmetry reflects an individual's inability to cope with environmental stress during trait development², Møller argues that preferences for symmetrical ornaments may have evolved because they enable females to choose high quality mates and so obtain "good genes" for their offspring¹.

Alternative explanations for the evolution and maintenance of tail symmetry are suggested by our work on the aerodynamics of bird tails³ showing that the lift generated by a tail depends on its angle of attack and the square of maximum continuous tail width. The aerodynamically optimum tail should therefore be triangular when spread and thus forked at rest. One drawback of a forked tail (like a swallow's) is that a high proportion of the lift that it produces is generated by its outermost feathers, and so any asymmetry in those feathers will greatly reduce lift (see figure). Furthermore, asymmetry will affect the distribution of lift across the tail and introduce large rolling and yawing forces which a bird could overcome only by flying with its tail at an angle. Therefore even a moderate degree of tail asymmetry will exert a profound effect on the manoeuvrability of male swallows (as Møller has already demonstrated experimentally⁴).

This has two important implications.

First, it suggests that symmetrical outer tail feathers may have evolved and been maintained primarily under natural rather than sexual selection. One reason for this is that the substantial reduction in agility associated with increased asymmetry is likely to have a considerable impact on the feeding efficiency of an aerial insectivore such as a swallow. Møller sought to refute this possibility by showing that manipulation of male tail symmetry does not affect the size attained by nestlings fed by males¹. But because tail elongation also has no effect on nestling size¹, but is known to reduce male foraging efficiency⁵, the size of nestlings is clearly not a reliable index of male feeding ability. If, instead, asymmetry does depress feeding efficiency substantially, it may well have a greater effect on male fitness than the 15–25% reduction in annual reproductive success attributed to female discrimination against males with artificially asymmetrical tails¹.

Second, the observed effects of tail symmetry on the mating success of male swallows need not necessarily arise through female preferences for symmetrical tails. For instance, the reduction in manoeuvrability caused by asymmetry may have substantial direct and indirect consequences for an individual's ability to compete intrasexually for mates. Again, evidence presented against this suggestion is unconvincing. Different symmetry treatments do not affect the average level of male–male aggression seen during three one-hour watches¹, but if such encounters are infrequent, the high sampling error associated with short watches may obscure real differences in aggression rates (mean values and ranges are not given). Moreover, no data are presented on the crucial issue of whether asymmetry treatments affect the



Effect of asymmetrical outer tail feathers on lift, calculated using a lifting surface model of tail aerodynamics³. Filled circles, a tail that is forked when at rest and asymmetrical when spread; open circles, weaker effect of asymmetry on the lift of a tail that is triangular at rest. Both tails have an apex spreading angle of 120°, and an angle of incidence to the local flow direction of 10°; outer rectrices are 100 mm long. The variation in asymmetry considered (relative to outer rectrix length) corresponds to the range in mean relative asymmetry seen across males of 15 species with ornamental tails⁶, and is far less than the maximum degree of asymmetry produced by Møller's manipulations. The maximum asymmetry of 10 mm introduces a 13% reduction in total lift generated by the forked tail. This relative loss would diminish if the same absolute asymmetry occurred in a longer forked tail (in which the outermost rectrices contribute relatively less to the maximum continuous span of the spread tail). This may explain the greater effect of asymmetry on the mating success of males with shortened rather than elongated tails (see Fig. 1, ref. 1).

outcome (rather than simply the rate) of agonistic interactions, particularly during territory establishment. Finally, the far-reaching consequences of tail asymmetry mean that insofar as female choice does determine male mating success, asymmetrical males may be discriminated against through a female preference for some general aspect of male athleticism, rather than tail symmetry *per se*. Thus while the adaptive importance of tail symmetry is evident^{1,3,4}, the nature of the selective pressures favouring symmetry remain unresolved.

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SIR — We question Møller's claim¹ that female barn swallows use male tail feather asymmetry as an indicator of genetic quality. He offers no direct support for this hypothesis. A likely function for elongated, symmetrical male tail feathers is that they indicate male experience and females use them to assess potential male parenting ability. Previously, Møller showed that male tail length correlates strongly with arrival date and age⁷,

which reflects experience, and that male barn swallows feed nestlings as much as or more than females⁵. Therefore, differences among males in ability to give care should influence female mating decisions. Møller now offers nonsignificant differences in offspring tarsus length and body weight among treatments as evidence against the hypothesis that tail length and symmetry affect foraging ability. This test fails to address the possibility that females use male tail feathers to predict parenting ability, and that differences in male parental care between treatments influence number, rather than size, of young fledged. To exclude nongenetic benefits to choosy females, unmanipulated tail feather length and symmetry must not correlate with male ability to provision and fledge young.

Møller's new results also contain several inconsistencies. Of particular concern are the small standard errors reported for pre-mating period and egg-laying date in each treatment compared with two earlier experiments on barn swallows^{8,9} that replicate the two asymmetry controls in which Møller¹ shortened or elongated tail feathers but did not alter their symmetry. For example, the pre-mating period for the shortened asymmetry control has a standard error only 7% of that reported previously⁸, despite similar sample sizes. Further, the two asymmetry controls do not contain sufficient unexplained variation to account for the effects of the treatments where both length and asymmetry were altered.

Møller states that all males within each treatment, including those with shortened tails and experimentally enhanced asymmetry, mated¹. This is at odds with his earlier 3-year study in which 33% of males with short tails failed to mate⁷. If females discriminate against asymmetric, short-tailed males, then more of the extreme experimental males should go unmated than unmanipulated males.

The experimental treatments altering tail feather asymmetry and length explain nearly all the variation in pre-mating period and egg-laying date. With such small errors within and large differences between treatments, we question how Møller could have assigned males at random to treatments as he states. The sample sizes he reports are not consistent with simultaneously assigning males to treatments in octets. If males were assigned to groups in order of arrival, then this could account for the low variation and contribute to differences between treatments.

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MØLLER REPLIES — Balmford and Thomas suggest that ornamental symmetry may have evolved and been maintained primarily under natural selection. This may not be particularly likely because the primary reason for the increased level of asymmetry and the pattern of asymmetry in secondary sexual characters is obviously sexual selection. They suggest that asymmetry may have a substantial direct fitness effect on females by affecting male food provisioning. Contrary to what one perhaps should expect, the most preferred males feed their nestlings less than the least preferred males¹⁰. In other words, females work harder if they have acquired a preferred male. This was also the case in the asymmetry experiment (A.P.M., manuscript submitted), and there is no net effect on offspring size because females compensate for their mates. The relationship between female parental care and male ornament size is a direct causal relationship as demonstrated by a tail manipulation experiment performed after mate acquisition (de Lope and Møller, manuscript submitted).

Balmford and Thomas also suggest that the effects of the experiment may have arisen due to male combat rather than female choice. I dismissed this possibility¹ although I gave no data for reasons of brevity. There is clearly no effect on male fighting. The frequency of fights is very high, and the sampling periods are of a sufficient duration as determined by repeatability and consistency analyses. The outcome of male fights was not considered because males almost always win (in more than 99% of cases) when fighting within their own territory due to site related dominance.

Borgia and Wilkinson suggest that females may have chosen males with symmetric and long tails for the direct fitness benefits. Males do provide a lot of parental care, but this cannot be the reason for female mate preferences. Females mated to preferred males provide relatively more, not relatively less care¹⁰. There is a direct (negative) causal relationship between parental care and male ornament size because a tail length experiment performed after mate acquisition demonstrated that females mated to males which suddenly had elongated tails provided relatively more parental care (de Lope and Møller, manuscript submitted).

Borgia and Wilkinson further suggest that the standard errors are only 7% of those reported from a previous experiment, but the previously reported data contained standard deviations, not standard errors. They also claim that the two treatments that retained natural asymmetry did not show greater unexplained variation than the treatments where both

length and asymmetry were changed. This is not the case because these explained 3 and 7% less of the variance. A larger fraction of males could not be unmated because there is a large between-year variation in the percentage unmated males which ranges from 2 to 25%, on average 12.5% (ref. 11). The percentage unmated males in 1991 was in fact very low, only 2%.

Finally, the sample sizes are claimed not to be consistent with simultaneous assignment of males to octets. A total of 12 complete octets were created since octet number 13 was only partly filled because no more unmated birds were available. Four birds were never resighted again after release. Males were not assigned to treatment groups in order of arrival since arrival date was unrelated to treatment ($F = 0.16$, d.f. = 7, 86, $P = 0.99$).

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Confusion of kingdoms

SIR — Field biologists are not the intended audience for most of the advertising appearing in *Nature*, but the illustrations occasionally draw our attention. Not always with the desired effect, however. In your 21 May 1992 issue, below a photograph of several gorgonians, we learn that "To produce the highest quality agarose, it is essential to begin with the best seaweed. . .".

Marine invertebrates comprise or dominate more than ¾ of the 30-plus phyla of multicellular animals currently extant; J. Mann in *News and Views* (*Nature* **358**, 540; 1992) outlines their considerable importance in natural products research. Yet how many biologists can identify these creatures to phylum or even kingdom? Not enough apparently.

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NATURE · VOL 359 · 8 OCTOBER 1992

The roots of nature

Robert Temple

History of Animals (3 volumes)*; Parts of Animals, Movement of Animals, Progression of Animals (1 volume)†; Generation of Animals (1 volume)‡. By Aristotle. Loeb Classical Library/Harvard University Press: 1937–1991. Each volume \$15.50, £10.50.

Enquiry into Plants, On Odours, On Weather-Signs (2 volumes)§; De Causis Plantarum (3 volumes)||. By Theophrastus. Loeb Classical Library/Harvard University Press: 1916–1990. Each volume \$15.50, £10.50.

Theophrastus of Eresus: Sources for his Life, Writings, Thought and Influence (2 volumes)¶. Edited by William Fortenbaugh et al. Brill: 1992. Pp. 465, 705. £50, \$100, Dfl. 175 (both volumes).

Theophrastus: His Psychological, Doxographical and Scientific Writings.** Edited by William Fortenbaugh and Dimitri Gutas. Transaction: 1992. Pp. 410. \$44.95.

WESTERN science was essentially founded by Aristotle and his pupil and lifetime colleague Theophrastus. But not until now, some two millennia later, have all their surviving zoological and botanical writings become available in decent texts and translation.

Aristotle's writings on zoology were the first great works of scientific observation. Although he considered the explanation of biological phenomena to be the ultimate goal, he recognized the primary importance of gathering data, cataloguing this in his *Historia*. The appearance of the final volume of this work, under the title *History of Animals*, brings to a close the Loeb Classical Library's project of publishing the entire surviving scientific canon of Aristotle in English with facing Greek text, a task that has taken 55 years to complete. And the final volume is the best, containing a spectacularly comprehensive index and the so-called Book X of the *History*, which was tacked on through scribal error centuries ago and has now been identified as Aristotle's separate treatise *On Failure to Generate*. It should be emphasized that the title *History of Animals* is itself a mistranslation — an accurate rendering would be *Information about Animals Obtained by Enquiry*. The work is in fact a magnificent, and often surprising, compilation of just that, relating to no fewer than 560 named species, the structure, physiology and behaviour of many of them described in great detail. Here we learn, for example,

that Aristotle had discovered the 'waggle dance' of bees, anticipating von Frisch by some 2,300 years.

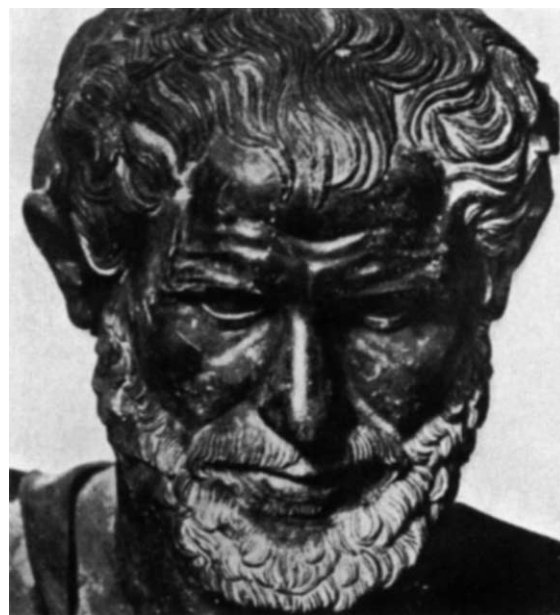
Aristotle was fanatically devoted to personal observation (made easier by the fact that he lived in a wide variety of localities in Greece, Asia Minor and the islands). When he had to rely on the reports of others, he did so with scepticism, often writing "but this has not been verified" or "this has not yet been properly observed". Even his information from exotic locations tended to come only from trusted sources, such as his nephew Callisthenes who accompanied Alexander the Great on his conquests. Alexander, whose tutor Aristotle had been, was a benefactor of Aristotle's studies; royal Macedonian funds subsidized the research institute, called the Lyceum, established by Aristotle at a former gymnasium in Athens in 335 BC. There, Aristotle founded the practice of team research:

botany and meteorology were delegated to Theophrastus, music to Aristoxenos, medicine to Diocles, history to Dicearchos, mathematics and astronomy to Eudemos, and so on. The purpose of the 'school' was evidently the construction of an encyclopaedic collection of knowledge about everything in the world, together with explanations if possible.

The unsung heroes of Aristotle's work were his assistants — beekeepers, eel-farmers, hunters, bird-catchers, midwives, fishermen, farmers, camel-drivers, elephant-drivers, horse-trainers, pigeon-keepers, peafowl-breeders, herdsmen, pig-keepers, shepherds, sponge-divers, drug-sellers and ordinary countrymen. He befriended them in complete defiance of social norms. His

zoological works are thus a compendium of what was known by these people in his time. He writes, for example, that "it is a very rare thing to find a female hyaena: a hunter told me that out of eleven hyaenas he caught only one was female". But perhaps most astonishing are his marine observations, which indicate that he hired divers to spy for him; he knew, for instance, that the grey mullet hides its head in the sand of the seabed in the belief that it is thus invisible.

Plenty of evidence of the empirical method exists in Aristotle's work. Indeed, there was originally a companion work to his *History*, entitled *Dissections*. Nearly as long as the *History*, this lost work, to which Aristotle refers constantly, consisted of many annotated dissection diagrams. It seems that Aristotle dissected 200–300 species, from seals to spiders, and although he was coy about admitting it overtly, human corpses. (The *History* itself originally contained simple explanatory diagrams.)



Aristotle: fanatically devoted to personal observation.

Aristotle's longest zoological work, *Close Investigations According to Kind* (*Exētasmenōn kata Genos*), seemed also to have vanished without trace. But I believe that its scattered contents remain and can be reconstituted. It led directly on from his psychological work *On the Soul* and was intended to 'explain' the phenomena of enquiry marshalled in the *History* in terms of causes, thereby elucidating the essential differences or characteristics of living things. It consisted in sequence of the eight short, scattered treatises now called *Parva Naturalia*, *Parts of Animals*, *Progression of Animals*, *Movement of Animals* and *Generation of Animals*††.

A correct understanding of Aristotle's terminology is the key to his methodo-

* Books I–III and IV–VI translated by A. L. Peck, pp. 239, 414; Books VII–X translated by D. M. Balme, prepared for publication by Allan Gotthelf, pp. 605.

† Translated by A. L. Peck and E. S. Forster; pp. 556.

‡ Translated by A. L. Peck; pp. 608.

§ Translated by A. Hort; pp. 475, 499.

|| Books I & II, Books III & IV and Books V & VI. Translated by B. Einarson and G. K. K. Link; pp. 361, 361, 465.

¶ Contains the complete fragments of Theophrastus in English translation: E. J. Brill, PO Box 900, 2300 PA Leiden, The Netherlands.

** Contains *Meteorology* and *On Fish*; Transaction Publishers, New Brunswick, New Jersey 08903, USA.

†† Aristotle's *On the Soul*, *Parva Naturalia* and *On Breath* are also published in the Loeb Classical Library Series by Harvard University Press (revised 1957, translated by W. S. Hett); pp. 528; \$15.50, £10.50.

logical approach in biology. Modern scholars have now established that the words *genos* and *eidos* should not be translated 'genus' and 'species', and to do so reduces Aristotle's zoology to a confused mass of apparent contradictions. The two terms as used by Aristotle were not classificatory but analytical. Aristotle never had taxonomy as an aim,

fascinating detail. More than 500 varieties of plant are described, and there are attempts at categorization and physiological theorizing (he was clear about the sexuality of plants, for example). The first volume deals with plants that grow of their own accord, whereas the two final volumes deal with "human ingenuity and contrivance" in relation to

plants, covering topics such as agriculture, viniculture, and pests and diseases of crops. Part of the introduction of the *Enquiry* seems to have been written by Aristotle, who then handed over the job to Theophrastus.

The causal book, more accurately translated as *Explanations of Plants*, originally contained two more books: Book VII became separated from the main work and for several centuries circulated under the title *On Wine and Olive Oil*, but, apart from five fragments, has now been lost; Book VIII also became separated, although most of it survives as *On Odours*.

But Theophrastus was not just a botanist — he wrote more than 200 treatises on an immense variety of topics, the complete fragments of which are collected and translated, for the first time, in two spectacular volumes just published by Brill. Here we find the separate treatises *On Types of Honey*, *On Flavours* and *On Fruits* and writings on physics, logic, metaphysics, mathematics, theology, zoology, botany, human physiology, ethics, politics, rhetoric, poetics, music and miscellanea. And the publication of Theophrastus's *Meteorology* should also be celebrated. It was thought that this work had been lost, but Hans Daiber found it in a maharajah's library in India in the 1980s, and the Arabic and Syriac texts and English translation now appear in an excellent volume published by Transaction. In *Meteorology*, we are treated to distinctions between different types of earthquakes and atmospheric phenomena (seven types of thunder are described). The volume also contains articles on sensory perception, vision, animal intelligence, the concept of place, the nature of the intellect and the opinions of other philosophers as dealt with by Theophrastus.

Whereas Aristotle died at the age of 63, Theophrastus lived into his 80s, and as many as 2,000 people eventually attended his lectures. In a fragment of a letter written late in his life, Theophrastus confesses that he cannot any longer

delay finishing his work — it is a curious irony that it took him nearly as long to complete his botanical works as it took the Loeb Library to publish them in English.

With these new books, zoologists, botanists, historians of science and philosophers will have an endless feast, and may also begin to understand the extraordinary symbiotic relationship that existed between Aristotle and Theophrastus. □

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Foundations of cosmology

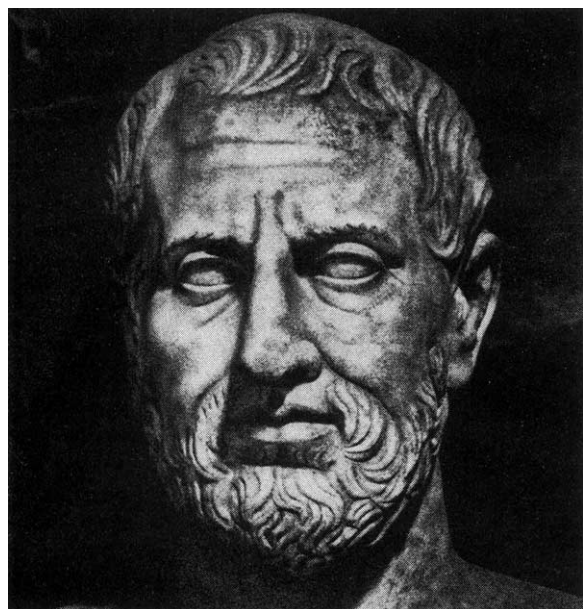
Joseph Silk

My Universe: Selected Reviews. By Ya. B. Zel'dovich. Harwood Academic: 1992. Pp. 252. £19, \$30 (pbk).

YAKOV Borisovich Zel'dovich was a cosmologist who never strayed far from physics. Indeed, he was also one of the world's leading physicists, with interests spanning many branches of physics. He wrote much of *My Universe* for someone defined as the pedestrian, a person (astronomer? chemist?) without a high-powered automobile but capable of walking whatever distance is required. Such a reader understands simple formulae, preferring them to vague descriptions, but would not follow complex calculations.

The chapter on cosmological field theory is a classic of its kind. Scalar fields are introduced in close analogy to newtonian gravity. We learn about the rise and fall, and finally the resurrection, of the scalar field, culminating in the Higgs field. Inflation is the best known manifestation of a Higgs field, even if the least susceptible to experimental testing. It is sad that the COBE (Cosmic Background Explorer) detection of fluctuations in the cosmic microwave background, a unique probe if not a proof of inflation, was announced less than five years after Zel'dovich's premature death.

Indeed, Zel'dovich pioneered exploration of the implications of the microwave background for cosmology. Together with his former student Rashid Sunyaev, he developed the theory of the shadowing of the background by hot gas in galaxy clusters, and a chapter in *My Universe* presents a lucid explanation of the Zel'dovich-Sunyaev effect. With another protégé, Alexandre Dolgov, he



Theophrastus: wrote on an immense variety of topics.

even by implication. He used *genos* to mean a category to which a definition of being attaches. An *eidos* is used to locate an entity within a *genos*; the entity's individual essence is then elucidated by a technique called "the listing of differences", by division into parts (a technical procedure derived from Aristotle's previous work on logic). And just in case we had any lingering taxonomic suspicions, Aristotle specifically states that one *genos* can contain others, and furthermore that an *eidos* can become a *genos*. To look backwards at his scheme through our taxonomic spectacles is therefore hopeless — his scientific work took place within a framework of thought quite distinct from that of the modern world.

Aristotle's closest associate was Theophrastus (c.371–c.287 BC), who succeeded him as head of the Lyceum. The two men seem to have been related, and may have been second cousins, as recorded by Ptolemy. Theophrastus shared Aristotle's general philosophical outlook (although he questioned and disagreed with some aspects), his commitment to observation and his range of interests. Most of Theophrastus's writings are now lost, but chief among surviving treatises are two botanical works: his *Historia* and *The Causes of Plants*, now published in the Loeb series as *Enquiry into Plants* and *De Causis Plantarum*, respectively.

Enquiry into Plants is packed full of



Monuments of Easter Island, painted in oil by William Hodges (c.1776). Hodges, official artist on Cook's second voyage to the Pacific, was one of the first artists to adopt the *plein-air* method of painting. The picture is taken from *Imagining the Pacific: In the Wake of the Cook Voyages* by B. Smith, which examines how science, topography and travel influenced artistic representation. Published by Yale University Press, \$70, £45.

describes why matter overwhelmingly dominates over antimatter in the observable Universe. And with another, Andrei Starobinsky, Zel'dovich lucidly accounts for the creation of the Universe from nothing, or more precisely, from almost nothing. The ultimate 'Theory of Everything' is needed to describe the first instants of the Universe, where all known physical laws break down, before we can understand what creation truly involves. Perhaps it is spontaneous creation from 'anything', as likely a precursor to the observable Universe as 'nothing'.

Although some of the articles that are translated from the original Russian are dated, the topics manage still to be topical. Mikhail Sazhin coauthors a chapter with Zel'dovich on gravitational waves in cosmology. This subject has been reinvigorated with the COBE measurement of fluctuations in the cosmic microwave background, one explanation of which is attributable to large-wavelength gravitational waves that are a relic from the inflationary epoch some 10^{-35} seconds after the Big Bang. If this turns out to be correct, one would reluctantly conclude that COBE tells us nothing about the density fluctuations that gave rise to large-scale structures such as galaxies and galaxy clusters.

Zel'dovich's love for cosmology is reflected by many pithy and personal

comments sprinkled throughout *My Universe*. Some are attributed to others ("cosmology is often in error but never in doubt"; Landau) or are about the bandwagon tendency of cosmological theorists to lay undue emphasis on visual impressions of cosmological data: ("Turner's paintings have affected England's climate: the numbers of clear evenings having brilliant sunsets over a rolling sea has increased dramatically"; Wilde). But some are his. On baryon asymmetry, he writes: "almighty God throwing dice for every single proton or antiproton would soon get tired with the astronomical number of particles. He could not make the asymmetry large enough." On high-brow theoreticians, he concedes that "the ultimate topic — the very birth of the universe — remains out of reach of even the highest brow", and on scalar fields, "once the genie has been let out of the bottle, he never wants to go back inside".

Those fortunate enough to have known Zel'dovich will enjoy these selected essays and reviews. All readers will delight in his colourful language, which loses little in translation and is a glowing testament to his lively and interactive personality. □

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Accidental tourist guides

Richard G. Vile

AIDS 91 Summary: A Practical Synopsis of the VII International Conference.

Edited by J. R. Berger et al. Philadelphia Sciences Group, 11232 Midlothian Turnpike, Suite 325, Richmond, Virginia 23235-9620, USA: 1992. Pp. 350. \$85 (pbk).

AIDS 1991: A Year in Review. Edited by F. Wong-Staal et al. Current Science: 1992. Pp. 270. £35, \$59.

To be well informed of the most important advances in AIDS research across the entire spectrum of basic scientific, clinical, social and political issues is a dream of many but is accomplished by few. Similarly, for any single, manageable publication to hope to span the same range would seem to be, at the very least, highly optimistic. These two volumes attempt to present a representative cross-section of the state of this particular art as it stood in mid-1991.

AIDS 91 Summary documents the proceedings of the Seventh International Conference on AIDS held in Florence in June 1991. The volume is divided into 10 chapters which reflect the breadth of the topics covered during the six days of the conference. Each chapter covers a different discipline ranging from the basic virology of the human immunodeficiency viruses (HIV) to the issues of education and counselling. Meeting reports can run the risk of presenting only disjointed pieces of information, but that is not a criticism that can be levelled here. The size of the conference ensured that there were sufficient talks to allow a smooth, logical progression through each chapter, and any gaps that may have arisen are more than adequately compensated for by editorial infilling and background information. Indeed, the overall effect is more one of a comprehensive textbook than a meeting report. As such, the volume provides a welcome opportunity for the reader to become broadly well-informed with a minimum of effort. But if it is to be used as a textbook, two points should be emphasized. First, there are no references to the primary literature to support the scientific research and opinions that are presented; the book cannot therefore be used as a portal to the vast scientific literature on AIDS that now exists without author-name searches on literature databases. Second, the findings that are reported and accredited to individual presenters are, by understandable necessity, rarely supported by any critical discussion. It would be misleading if the uninitiated

reader were to gain the impression that AIDS research is pleasantly free from controversy.

A more subjective but critically presented review of similar completeness is provided by *AIDS 1991*. This is the latest volume in a now well established and popular series of annual reviews that form a supplement to the journal *AIDS*. It is certainly the route to take into the literature of any specific topic, whether it be basic molecular biology or more social topics such as the role of law in AIDS policy. For each of the five main topic areas chosen (virology; epidemiology; vaccines and immunology; clinical treatment; social, cultural and political aspects), chapters are contributed by many of the most respected workers and are united by an editorial overview. Each chapter reviews the primary literature in a readily accessible way, making further investigation easy. This is certainly the reference volume that will prove of greatest value to the researcher.

Even the most conscientious conference devotee is hard pressed to attend all the relevant, often concurrent, sessions — especially when the conference is in Florence. *AIDS 91 Summary* provides an opportunity for those who attended to compensate for areas they missed and the chance for those who were not at the conference to review the proceedings. For others just entering the field or realistic enough to admit that, much as they would like to follow many more areas in depth, time rarely permits them to do so, the volume provides a useful all-round summary, albeit on a fairly superficial and uncritical level. If interest is sparked and further depth required, however, it is reassuring to have on hand review editions such as *AIDS 1991*. Of course, it could be argued (especially by lovers of David Lodge's novels such as *Small World*) that these review volumes may provide hard-pressed grant-giving bodies with a persuasive argument for keeping eager conference goers at home, rather than allowing them to sample the splendours of cities such as Florence. To counter such an outrageous suggestion, it is important to point out that in Science, as in Art, it is infinitely more instructive to view Michelangelo's *David* at first-hand rather than to be forced to assess its merits second-hand from a guide book. □

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■ *Acquired Immune Deficiency Syndrome: Biological, Medical, Social, and Legal Issues* by Gerald J. Stine is a new overview volume that was received too late for inclusion in the above review. Aimed at health professionals, educators and university students, the book is published by Prentice Hall, price \$40.

Love potions

David Crews

Chemical Communication: The Language of Pheromones. By William G. Agosta. W. H. Freeman: 1992. Pp. 179. £17.95, \$32.95.

LOVE is the best known pheromone language, with males or females, or both, producing compounds that signal their readiness to mate. The message can be exceptionally subtle. A change in a single chemical bond can nullify an otherwise aphrodisiac odour or a particular arrangement of atoms can keep two closely related individuals from mistakenly mating as they pass in the night. But the chemical senses communicate much more; pheromones can identify individuals, communicate danger, serve as navigational aids to animals and cells, signal the appropriate time to hatch or regulate development, impart where to live and when to aggregate, and even deceive.

A once popular distinction was that animals communicate by sights and sounds whereas cells communicate by chemicals. We know now that many animals can sometimes sense a few molecules of a particular odour produced by another individual and that these chemicals can dominate interactions and regulate physiology. In just 50 years, the study of pheromones has developed into one of the great success stories of biology. An enormous amount has been learned about the manufacture of pheromones, their physical structure and functional properties, as well as how pheromones are perceived and exert their actions. These advances have had a considerable effect on pest control, given us clues about bonding between mothers and infants, aided in fertility research and, of course, motivated the perfume industry. William Agosta neatly summarizes our understanding, capturing the excitement of pheromones without oversimplifying the complexity of this interdisciplinary field.

The chemical senses of humans have been assumed to be poorly developed, yet the recent discovery that adult humans possess a sensory structure known as the vomeronasal organ raises new questions about this generality. The rapid advances in this new field are due to its allure to organic chemists, physiologists, behaviourists, ecologists and, most recently, molecular biologists. Pheromone research is therefore not done by individuals so much as by teams of scientists often representing a mixture of talents as complex as the ingredients of the compounds they study.

Blending the information in an enjoy-

able synthesis, Agosta introduces the reader to this fascinating field of inquiry. Work began, and continues to focus, on glandular products. But some pheromones saturate the skin and have no specific glandular source. Here J.-M. Jallon has recently shown how the sexual mosaics produced for molecular genetics can be used to locate the source of cuticular pheromones in fruitflies.

Another fascinating problem is the relation between pheromones and hormones. One school of thought holds that pheromones served initially as communicators between single-celled organisms and, with the evolution of multicellularity, became hormones. Others have argued persuasively that pheromones were hormones first and only later assumed secondary functions as communicators between individuals because they accurately reflected specific physiological states. Both have probably occurred. An example is provided by the elegant work of N. Stacey with goldfish, showing how chemicals serve as both hormones and pheromones. In the goldfish, two reproductive hormones, one a steroid and the other a prostaglandin, assume roles as pheromones as they are excreted by the female. As the male detects these compounds, milt production is increased and courtship behaviour elicited. So pheromones can have several different actions, serving to activate specific behaviours (releasing pheromones), determine how an individual will develop and regulate physiological processes (priming pheromones) or communicate motivational state (signalling pheromones).

Chemical Communication is written and presented in the style of *Scientific American* articles that feature a single topic and are rich in detail with fascinating examples and handsome illustrations. Agosta shows how pheromones are molecular messages that are woven into a language in a species and even, occasionally, between species. It is informative and enjoyable and written so as to appeal to everyone from advanced high school or undergraduate students to specialists in the field. □

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New in paperback

■ *Last Animals at the Zoo: How Mass Extinction Can Be Stopped* by Colin Tudge. Reviewed in *Nature* **354**, 195 (1991).

■ *The Shadows of Creation: Dark Matter and the Structure of the Universe* by Michael Riordan and David N. Schramm, with a forward by Stephen Hawking. Reviewed in *Nature* **352**, 677 (1991).

Spatial and temporal variability in the geochemistry of basalts from the East Pacific Rise

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Closely spaced sampling of basalts around the East Pacific Rise, made possible by a new sampling technique, reveals spatial patterns of change in the composition of the sea floor. The patterns demonstrate the scale and magnitude of temporal variability in the basalt chemistry, and suggest that the characteristic length scale of petrological segmentation can vary over time from 15 to >40 km along a single section of ridge. Patterns of basalt composition on the sea floor reveal the history of the ocean ridge with much finer resolution than do sea-floor spreading magnetic anomalies.

STUDIES of most young volcanoes are restricted to a limited window of time, because new volcanic outpourings cover older flows. At mid-ocean ridges, however, the spreading of the sea floor transports older volcanic rock sideways, preserving a record of the temporal changes. The magnetic signature of this record on the ocean floor has provided evidence for sea-floor spreading¹, ridge jumps², ridge propagation³, and the geometry and history of ridge segmentation⁴. The composition of the basaltic rocks might also record changes at the ridge, possibly on an order of magnitude finer timescale than the magnetic sea-floor spreading 'stripes'.

Regional studies on the East Pacific Rise (EPR) have shown that geochemical segmentation of the ridge axis corresponds with structural segmentation⁵⁻⁶, characteristics of the seismic magma chamber reflector⁷⁻⁹ and crustal velocity structure¹⁰, suggesting that segmentation is linked to offsets of the axial magma systems. Off-axis geophysical surveys¹¹⁻¹³ have explored the evolution of structural offsets of the EPR, but the evolution of basalt geochemical segmentation is largely unexplored. One way of examining temporal variability in volcanism is to look at the vertical stratigraphic record accessible by crustal drilling¹⁴, but this is difficult and expensive. A study near 21° N (ref. 15) showed that changes in sea-floor basalt composition with time could produce a symmetrical zonation from the axis out to 2 km on either side. But to assess the potential of petrology for mapping temporal evolution and its relation to segmentation, it was necessary to investigate a greater length of ridge out to a greater distance from the axis.

Our study area covers 55 km of the EPR from 12° 00' N to 12° 30' N (Fig. 1), out to 10 km from the axis, or a crustal age equivalent to about 200,000 years (based on a full spreading rate of 10.7 cm yr⁻¹; ref. 11). The nearest large offsets are overlapping spreading centres (OSCs) at 11° 45' N (a 7.5-km offset) and at 12° 37' N (1.8 km)¹⁶. Between the OSCs are three smaller offsets, called devals¹⁷. Devals are small deviations from axial linearity, and correspond to offsets of the axial fissure swarm by several hundred metres¹⁷⁻²⁰. The 12° 09' deval is a 300-m, right-lateral offset of the axial fissure zone. At the 12° 16'–12° 20' deval, the axial fissure zones of adjacent segments overlap left-laterally. North of the 12° 28' deval, the axial fissure zone breaks into a series of right-stepping, *en echelon* strands which continue to the 12° 37' OSC. Thus the study region includes two deval-bounded segments (12° 28' to 12° 16', and 12° 20' to 12° 09') and a portion of a third (south of 12° 09'). We refer to them as the northern, central and southern segments.

Previous dredges along the axis recovered normal, depleted mid-ocean-ridge basalts (N-MORB) in the northern and southern segments. The central segment yielded both N-MORB and basalts enriched in incompatible elements such as Ba and K (T-MORB)⁶. Segmentation of the geochemical signature therefore seemed to correlate with the structural segmentation. The relationship of the deval-bounded segmentation to the magmatic system was, however, ambiguous because the central segment was characterized by greater diversity, rather than by a coherent, geochemically distinct composition.

In part, this ambiguity arose because dredging has limited spatial resolution. Small-scale spatial variations therefore could not be resolved. To overcome this problem we developed a new sampling technique, 'rock coring', similar to a method used on HMS *Challenger* in the nineteenth century^{21,22}. The rock core assembly consisted of a sharpened core tip filled with wax and attached to a gravity core barrel. The core tip slammed into the glassy surface on the sea floor, and glass chips were recovered in the wax. Rock coring was 3–5 times faster per sampling station than dredging, with an order of magnitude better spatial resolution.

This technique allowed us to study the temporal variability in EPR basalt compositions. Here we show how the intensive sampling reveals patterns in the composition of the sea floor that result from evolution of the ridge in space and time. The patterns indicate that the scale of temporal variability is short, that parental magmas controlling crustal composition change rapidly and that the length scale of petrological segmentation along the ridge can vary over short (10⁴-yr) time intervals.

Spatial systematics of 12° N basalts

During our survey on the RV *Thomas Washington*, 127 dredge and rock core stations (Fig. 1) were located with reference to SeaBeam bathymetry²³, the Global Positioning System satellites and bottom-moored acoustic transponder nets. The sampling strategy was guided by shipboard chemical analyses of basaltic glasses using direct-current plasma spectrometry. Samples were subsequently analysed by electron microprobe at the Lamont-Doherty Geological Observatory. Representative analyses are reported in Table 1. This sample set was augmented by 12 earlier dredges along the axis⁶ (Fig. 1). In addition, previously collected SeaMARC I side-looking sonar data^{24,25}, plus new camera and video tows, provided a geological context for some of the samples.

In the 12° N region, the EPR axial block consists of a central

summit depression bounded by two 'axial highs' and steeply sloping 'flanks' (Fig. 1c). The region outside the main break in slope is 'off-axis'. The depression is a fault-bounded graben, present along the entire length of the study area, although its relief and width vary. The axial highs are not uniform along strike, and there are occasionally bulges to one side or the other, which have the appearance of exceptional volcanic outpourings.

The clearest geochemical distinction that can be made among the 12° N samples is between normal mid-ocean-ridge basalts (N-MORB) and those transitional (T-MORB) to the enriched basalts found near hotspots²⁶. The locations of these two sample types are identified in Fig. 1 by filled symbols for N-MORB and open symbols for T-MORB. T-MORB have fairly high incompatible element contents (defined here as $\text{Ba}/\text{TiO}_2 > 10$ or $\text{K}_2\text{O}/\text{TiO}_2 > 0.09$), and distinctive high Al_2O_3 and Na_2O and low FeO^* (total iron as FeO). The contrast between these groups dominates the geochemical signal. In fact, the initial definition of deval-bounded petrological segmentation was based on along-strike distribution of N-MORB and T-MORB⁶. Careful examination of the complete data set, however, shows that there are two main forms of geochemical variation among the EPR basalts: the variation from 'pure' N-MORB to T-MORB, and a variation within the N-MORB alone. In view of these systematics, we first examine the data from each group separately.

Zonation of N-MORB compositions. Although all the N-MORB have low $\text{K}_2\text{O}/\text{TiO}_2$ and Ba/TiO_2 ratios (see Table 1), their

major element compositions vary considerably. Different groups of samples lie along separate liquid lines of descent, as indicated by their different concentrations of FeO^* , TiO_2 and CaO for a given MgO content (Fig. 2). MgO is a proxy for temperature, so to remove cooling effects we have normalized the data to a constant MgO content of 7.3%, which is the average for the entire data set. Once the N-MORB data are considered separately from the T-MORB, it is clear that the variations in FeO^* , TiO_2 and CaO for the N-MORB correlate.

To bring out subtle patterns in N-MORB spatial variability, we have combined the normalized values for TiO_2 and FeO^* ($\text{Ti}_{7.3}$ and $\text{Fe}_{7.3}$) into a single function, $\text{TiFe}_{7.3}$, illustrated in Fig. 2. (See figure legend for calculation method.) The analytical error in $\text{TiFe}_{7.3}$ for any five-point analysis is ± 0.4 (2σ), so samples within that range may lie along similar liquid lines of descent. These variations cannot be attributed to mixing with a T-MORB component because the N-MORB/T-MORB contrast has different systematics from the low- $\text{TiFe}_{7.3}$ /high- $\text{TiFe}_{7.3}$ contrast.

The largest variations in $\text{TiFe}_{7.3}$ occur normal to the ridge axis, with the highest values in the axial graben and a progressive change to lower values off-axis (Fig. 2d, e and Fig. 3). The mean $\text{TiFe}_{7.3}$ decreases progressively (although not monotonically) from 0.52 for samples recovered from the axial graben to -0.12 for samples recovered 3–8 km off axis. All of the samples 6–8 km off-axis have lower $\text{TiFe}_{7.3}$ values than the mean of the graben

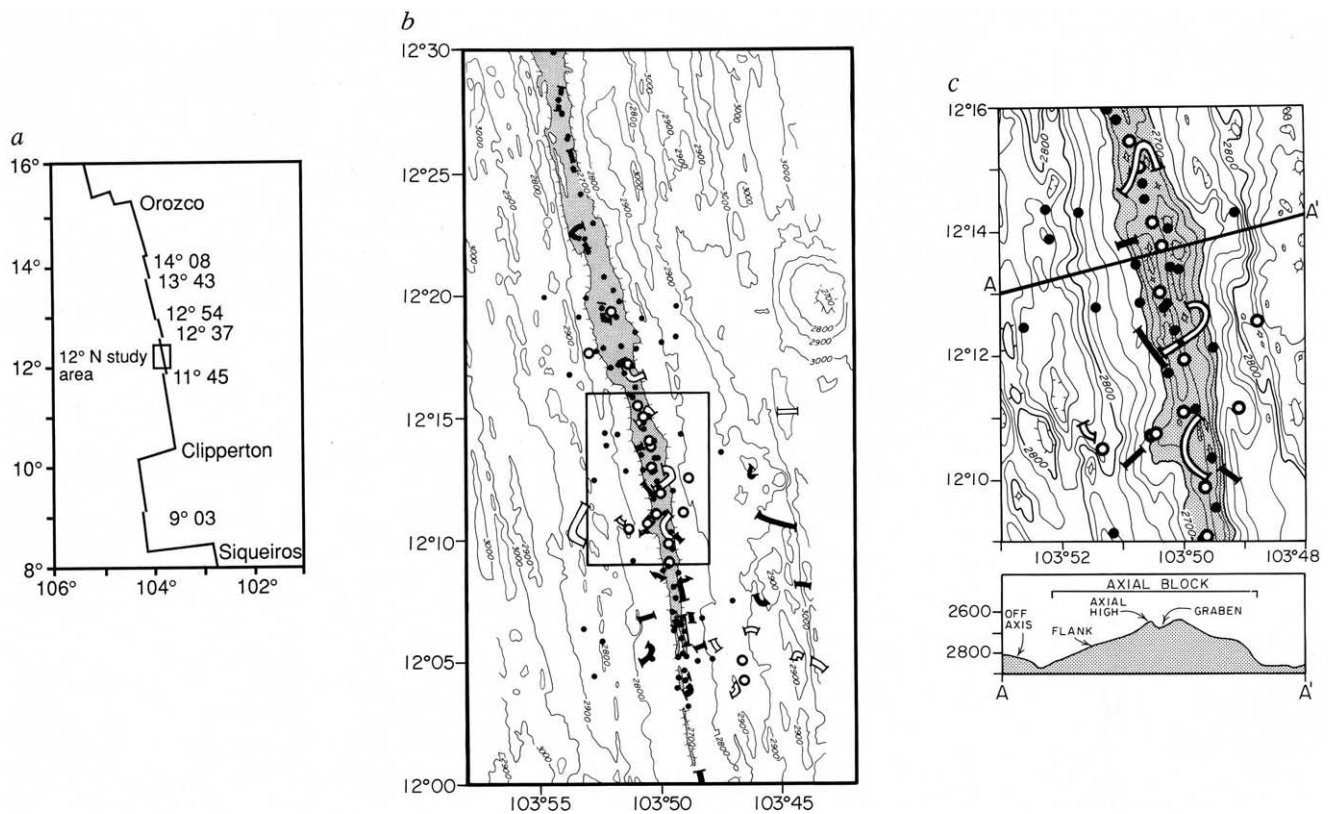


FIG. 1 Location and bathymetry of the 12°N survey area. *a*, East Pacific Rise, with major offsets of the ridge axis: transform faults (Orozco, Clipperton, Siqueiros) and overlapping spreading centres (for example at 14° 08'). The box at 12° N indicates the region shown in *b*, the 12° N survey area, with the distribution of dredge and rock core sample locations from this cruise and locations of earlier dredges from Langmuir *et al.*⁶ (along the axis at 12° 00', 12° 04', 12° 06', 12° 07.5', 12° 10.5', 12° 12.5', 12° 15', 12° 16.5', 12° 19', 12° 22', 12° 25' and 12° 28' N). Locations are shown on a Sea Beam bathymetric map (after Monti *et al.*²³), at 100-m contour intervals, and the region along the ridge axis shallower than 2,700 m is shaded. Circles represent rock cores, and lines terminated by bars are dredge locations. Filled symbols indicate locations with N-MORB basalts only. Open circles

indicate T-MORB rock cores, and open dredge symbols identify dredges that recovered both T-MORB and N-MORB (except VE-30 at 12° 05.3' N, 103° 44.4' W, which recovered T-MORB only). Symbol size greatly exaggerates the uncertainty of rock core sample locations (believed to be accurate to 150 m or better). *c*, Detailed portion of the map in *b*, showing the central segment of the ridge axis. Contour interval is 20 m. Samples are from the 1989 cruise, with the exception of three earlier dredges across the axis⁶ at 12° 10.5', 12° 12.5' and 12° 15'. The bathymetric cross-section at 12° 13'–12° 14' (based on 10-m contour data²³) illustrates the terms used in the text to describe typical morphological elements of the 12° N ridge axis. Vertical exaggeration is $\times 6$.

samples (Fig. 4d). The first-order symmetry of this pattern around the 12° N ridge axis is consistent with a gradual change in the chemistry of the lavas erupting at the ridge axis, over many generations of magma supply and eruption.

The detailed nature of this change varies along the EPR. Figure 4 shows the variation of N-MORB composition for several slices of sea floor at increasing distances from the ridge axis. The overall trend of decreasing $\text{TiFe}_{7.3}$ with increasing distance is again clear, but each off-axis slice shows a different petrological segmentation. The interval farthest from the axis (Fig. 4d) shows three distinct clusters of $\text{TiFe}_{7.3}$ values. In the next closest interval to the axis (1–3 km off axis, Fig. 4c) the data are variable and represent several different magma types. This may result from higher- $\text{TiFe}_{7.3}$ lavas that flowed up to several kilometres west of the axis, over lower- $\text{TiFe}_{7.3}$ regions of a single magma type (12° 08' and 12° 14'; see Fig. 3). The lavas covering the axial highs and flanks may also be related to a single parental magma type, with virtually all samples over a distance of 40 km along axis on a similar liquid line of descent (Fig. 4b). Within the graben, samples with $\text{TiFe}_{7.3}$ similar to the axial highs were collected at two of the devals and at 12° 03' to 12° 04'. High- $\text{TiFe}_{7.3}$ samples fill the rest of the graben (Fig. 4a).

Among graben samples, the lower- $\text{TiFe}_{7.3}$ samples may be older lavas not yet covered by higher- $\text{TiFe}_{7.3}$ flows from a new magma pulse. Data from the northern segment support this suggestion. Five rock cores from the central 7.5-km section of the graben have identical, high- $\text{TiFe}_{7.3}$ compositions within analytical error (Figs. 3, 4), suggesting that a single eruption or eruption episode may have flooded this 7.5-km stretch of the graben floor. On the adjacent axial highs, and at the two distal ends of the graben in this segment, lower- $\text{TiFe}_{7.3}$ lavas occur. The petrological pattern may therefore be interpreted as a temporal one with new eruptions in the central portions of segments, away from devals which mark the boundaries and the least active parts of the crustal magmatic systems.

The regularities in $\text{TiFe}_{7.3}$ can also be examined in a map view of the data (Fig. 3). The N-MORB data show a systematic change in composition, as a progressive trend from low to high $\text{TiFe}_{7.3}$ with decreasing distance from the axis, but the pattern of petrological segmentation along strike varies at different distances from the axis.

T-MORB distribution. The T-MORB occur mainly in two geographical clusters with characteristic Ba/TiO_2 : one along the

axial block of the central segment ($\text{Ba}/\text{TiO}_2 = 10.5\text{--}12.5$), and another east of the axis between 12° 03' N and 12° 07' N ($\text{Ba}/\text{TiO}_2 = 17.0\text{--}19.0$). There are also apparently isolated occurrences of T-MORB, particularly at the latitude of the central segment. Three of these have by far the highest Ba/TiO_2 (~60) recovered from the entire region. Similarly enriched basalts occur just north of this area, near the OSC at 12° 37' (ref. 6).

There is a possibility of geochemical asymmetry, because T-MORB were recovered off-axis from the southeast but not the southwest part of the study area. The southeast cluster occupies a 40 km² region, including stations where both T-MORB and N-MORB were recovered. On the other side of the EPR axis, symmetrical to this region, we recovered only N-MORB in two rock cores and a dredge, but more data are needed to define the situation conclusively.

Along the axial block, the locations of T-MORBs are systematic with respect to the current devol-bounded structural segmentation. T-MORB are confined to the central segment graben and the axial high bulge at 12° 10' (Fig. 1); none have been found in the other graben segments or elsewhere on the axial highs and flanks. Within the graben of the central segment, however, there is no spatial organization of the N-MORB and T-MORB. The petrological variability in the graben may instead be temporally organized. Sea-floor photographs collected during the cruise reveal an age range within the graben, and the Sea-MARC I images show younger flows which cover older fissures. One camera tow crossed such a region, and photographed a sheet flow younger than the surrounding pillow and lobate terrain. Rock cores recovered T-MORB glass from this sheet flow and N-MORB from the older terrain. At the axial high bulge at 12° 10', where T-MORB also occur, sea-floor photographs showed flows with anomalously light sediment cover (anomalously young). This evidence, combined with the fact that T-MORB were not recovered elsewhere on the older axial highs, suggests that T-MORB may be the youngest phase of volcanism. These data also demonstrate that considerable petrological variability can take place within a single segment in the time span represented by the graben itself.

Possible origins of the N-MORB systematics

The N-MORB systematics have both chemical and spatial aspects. The origins of the variations in $\text{TiFe}_{7.3}$ require more detailed modelling and discussion than is possible here, so

TABLE 1 Representative analyses of basalt glasses

Sample	Latitude	Longitude	Depth	Setting	Type	SiO ₂	TiO ₂	Al ₂ O ₃	FeO*	MnO	MgO	CaO	Na ₂ O	K ₂ O	P ₂ O ₅	Sum	Ba	Sr	TiFe _{7.3}
RC127	12° 26.50'	103° 53.70'	2,660	axial high	N	50.46	2.03	13.74	12.08	0.21	6.89	11.23	2.80	0.10	0.22	99.76			0.095
RC131	12° 26.40'	103° 53.75'	2,670	axial graben	N	50.67	2.13	13.46	12.45	0.21	6.82	10.98	2.66	0.13	0.21	99.71	10.8	95	0.406
RC116	12° 21.78'	103° 52.92'	2,680	axial high	N	50.25	1.71	14.48	10.84	0.23	7.56	11.71	2.73	0.08	0.19	99.78			0.084
RC72	12° 19.34'	103° 51.98'	2,660	axial graben	T	50.85	2.38	13.75	12.08	0.21	6.47	10.58	2.95	0.24	0.26	99.78	23.4	141	—
RC152	12° 19.06'	103° 53.12'	2,765	flank	N	50.65	2.05	13.92	11.88	0.21	6.73	11.17	2.88	0.12	0.20	99.82	8.6	108	-0.927
RC123	12° 17.08'	103° 51.08'	2,670	flank	N	50.53	2.08	13.67	12.25	0.21	6.81	10.97	2.87	0.15	0.22	99.76	10.5	107	0.116
RC136	12° 14.15'	103° 50.50'	2,670	axial graben	T	50.60	2.16	14.27	10.83	0.21	7.00	11.19	3.03	0.31	0.26	99.86	23.9	170	—
RC37	12° 13.37'	103° 50.07'	2,640	axial high	N	50.26	1.85	14.08	11.23	0.19	7.45	11.62	2.73	0.12	0.21	99.73	8.7	115	0.402
VE3-B	12° 12.6–13.0'	103° 46.1–46.2'	2,820–2,750	off axis	N	50.71	1.08	14.68	9.41	0.18	8.53	12.79	2.16	0.03	0.18	99.76	3.9	71	0.244
RC138	12° 12.20'	103° 50.04'	2,675	axial graben	N	50.03	1.21	15.72	9.04	0.14	8.80	12.41	2.33	0.07	0.19	99.93			0.770
RC139	12° 11.12'	103° 49.80'	2,680	axial graben	N	50.63	2.04	13.59	12.15	0.22	7.17	11.24	2.43	0.10	0.22	99.81	8.6	90	0.907
RC50	12° 10.50'	103° 51.32'	2,820	off axis	T	50.09	1.70	16.16	8.87	0.17	7.50	11.13	3.11	0.64	0.29	99.67	100	246	—
RC15	12° 08.10'	103° 49.47'	2,695	axial graben	N	50.63	2.17	13.65	12.49	0.21	7.02	10.99	2.52	0.09	0.21	99.96	13.6	96	1.126
VE25	12° 08.1–08.2'	103° 43.8–44.4'	3,145–2,900	off axis	N	50.59	1.88	14.14	11.17	0.22	7.10	11.38	2.95	0.14	0.22	99.76	11.9	122	-0.526
RC65	12° 06.77'	103° 48.29'	2,790	flank	N	50.67	1.49	14.38	10.23	0.18	7.82	12.16	2.61	0.08	0.19	99.82	5.7	98	-0.162
VE8-B	12° 06.7–07.0'	103° 50.9–50.1'	2,790–2,750	off axis	N	50.41	1.39	14.72	9.83	0.20	8.23	12.23	2.51	0.07	0.16	99.75	5.8	99	0.339
RC17	12° 06.53'	103° 49.15'	2,680	axial high	N	50.36	2.10	13.78	12.11	0.21	7.00	11.08	2.80	0.12	0.22	99.79			0.517
RC145	12° 05.85'	103° 52.40'	2,800	off axis	N	51.00	1.73	13.87	11.03	0.20	7.33	11.78	2.55	0.12	0.19	99.78	7.6	98	-0.627
VE23-B	12° 04.1–03.9'	103° 47.0–47.1'	2,900–2,840	off axis	T	49.05	2.04	16.28	9.51	0.19	7.47	11.09	3.54	0.33	0.26	99.74	37.2	220	—
RC32	12° 03.20'	103° 48.85'	2,715	axial graben	N	50.71	2.08	13.99	11.30	0.20	7.11	11.24	2.71	0.16	0.22	99.72	14.2	118	-0.069

Samples are all from the 12° N study area. Samples labelled RC are rock cores, VE are dredges. For dredges, the starting position and depth are listed first. The 'type' of sample is either N-MORB or T-MORB (see text). The major element data are electron microprobe analyses done at the Lamont-Doherty Geological Observatory; each composition is an average of at least five points, and all analyses are normalized to the JDF-D2 Lamont glass standard. Analytical precision of the microprobe data, as 2 standard deviations of the mean for a five-point average: SiO₂ 0.25, TiO₂ 0.04, Al₂O₃ 0.13, FeO* 0.18, MnO 0.03, MgO 0.13, CaO 0.13, Na₂O 0.10, K₂O 0.03, P₂O₅ 0.02. The calculation of $\text{TiFe}_{7.3}$ values for N-MORB samples is described in Fig. 2. Ba and Sr analyses were done at sea by direct-current plasma spectrometry. Precision as 1 standard deviation: Ba 2.5%, Sr 2.0%.

instead we emphasize the implications of the spatial systematics.

It is remarkable that the petrological variability of N-MORB is spatially organized, because many complexities could lead to a chaotic distribution of chemical compositions. These complexities include off-axis volcanism, large outpourings of lava that overflow the graben, transport of magma along many kilometres of rift zone before eruption and small ridge jumps^{10,27-30}. Irregularities in the spatial pattern in Fig. 3 may result from such events. Nonetheless, the data as a whole demonstrate a spatial coherence that must be caused by specific processes.

One option is to assume that the age of each sample is equivalent to the idealized crustal spreading age. In this case, the spatial variation in N-MORB chemistry would reflect progressive change through time in the axial magma compositions. One might then use the map of basalt composition to infer the history of petrological segmentation of the ridge axis.

Alternatively, off-axis volcanism may contribute significantly to the pattern. As an extreme view, one might consider that high-TiFe_{7.3} magmas are preferentially erupted in the axial graben, and that abundant off-axis volcanism covers them with progressively lower-TiFe_{7.3} lavas. This would create a regular stratigraphy off-axis, of low-TiFe_{7.3} flows at the surface and higher-TiFe_{7.3} flows at depth. Low-TiFe_{7.3} flows occur on-axis along nearby portions of the EPR⁶, however, so these lavas are evidently not restricted to off-axis occurrences. The central question is the extent of off-axis volcanism. We have suggested that some details in the compositional map are related to off-axis eruptions or to large flows originating at the axis (Fig. 3). Younger volcanics were identified in photographs at two locations outside the graben, including the axial high bulge at 12° 10' N. But aside from these exceptions, the camera and SeaMARC data show sediment cover increasing with distance

from the axial graben. Furthermore, all off-axis dredges recovered samples that are visibly much older than samples from the axis. Other investigators have also noted the lack of evidence for abundant off-axis eruptions²⁹⁻³². Off-axis eruptions may perturb the TiFe_{7.3} pattern, but they probably do not occur on a scale large enough to cause the overall spatial systematics.

Acceptance of the temporal hypothesis has certain implications. The first is a definition of the timescale on which the long-wavelength petrological signal operates. If one assumes idealized sea-floor spreading, with eruptions confined to the axis, then the ages of the basalts can be estimated from the half-spreading rate. Samples out to 1 km from the graben (axial highs and flanks) would represent the time back to ~19,000 yr ago; 1–3 km off axis represents ~19,000–56,000 yr; 3–6 km represents 56,000–112,000 yr; and 8 km represents ~150,000 yr (Fig. 4).

The range in TiFe_{7.3} in the 12° N data is equivalent to the entire range among N-MORB along the EPR axis from 5° N to 14° N^{6,25}. Assuming that those samples from 1,000 km of the present-day EPR axis define the compositional range that has erupted in the past, the 12° N pattern of decreasing TiFe_{7.3} from the graben away from the axis probably does not continue much beyond the off-axis edge of our survey, and the TiFe_{7.3} of magmas at the axis has probably reached a maximum. Thus the 12° N basalt compositions, from a 150,000-yr time period, may define roughly one-half of a petrological cycle in this region.

A second inference is substantial variation in the lengths of petrological segments through time. We define petrological segments as lengths of ridge where the basalt compositions may be derived from similar parental magmas by crystallization and differentiation^{6,33}. Currently, the graben shows petrological segments with lengths of 10–20 km, confirmed by the restriction of

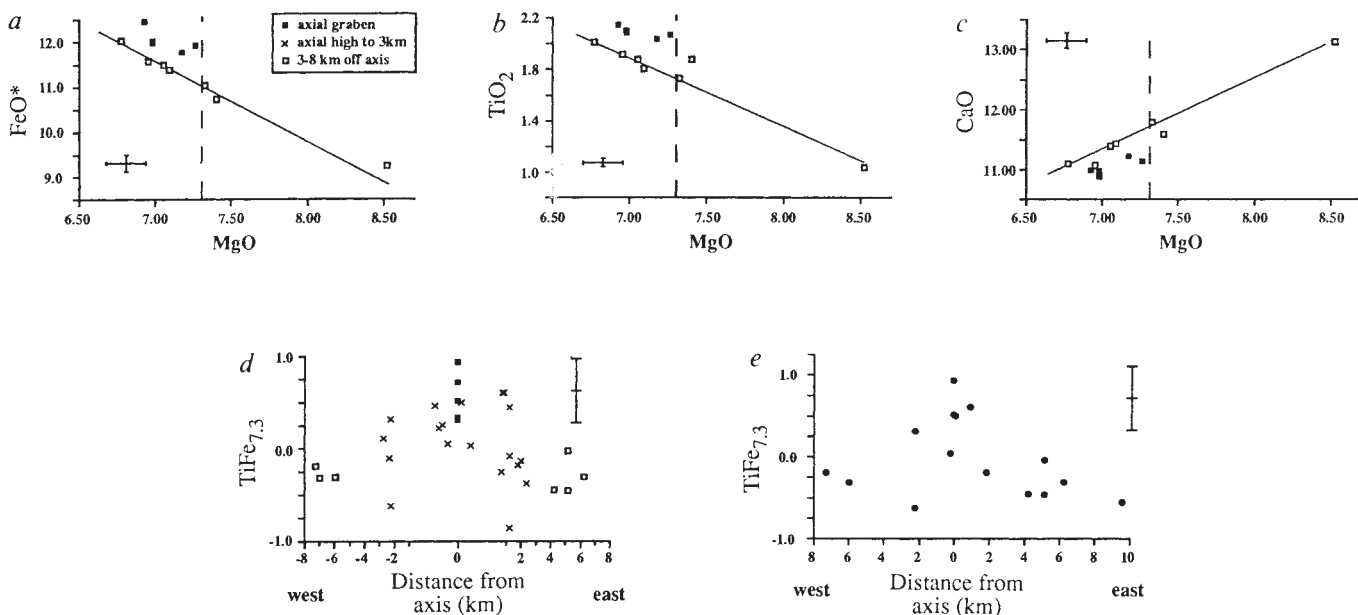


FIG. 2 *a-c*, Temporal differences in normal basalt (N-MORB) chemistry from 12° 04.3' N to 12° 08.0' N, a subset of the 12° N data. The size of this region was chosen to include enough samples to define the trends in these plots, illustrating trends in the full data set. Samples from the axial graben have higher FeO* and TiO₂, and lower CaO, than the basalts 3–8 km off-axis. For clarity, samples between the graben and 3 km off axis are not shown; see *d*. The data are electron microprobe glass analyses, and each is an average of at least five points. Error bars show two standard deviations from the mean, for an individual five-point average. *d*, Overall chemical trend through time, expressed by TiFe_{7.3}, of the samples in *a*, *b* and *c*. Distance

from the ridge axis is measured along flowlines. The scale at the axis (0 to 1 km) is enlarged to display the chemical difference between the graben and axial highs. TiFe_{7.3} is a function of FeO* and TiO₂ which have been regressed along the slope of the data to their values at MgO = 7.3%. (Slopes: FeO* = 1.8, TiO₂ = 0.53.) The average Fe_{7.3} is 11.16 and average Ti_{7.3} is 1.90. TiFe_{7.3} is calculated as (Fe_{7.3} – 11.16) + 1.5(Ti_{7.3} – 1.90). Ti_{7.3} is weighted more heavily to compensate for its smaller range in the N-MORB data set. *e*, Variation in TiFe_{7.3} along the narrow flowline for which we have the most data: a 1-mile-wide band beginning west of the axis at 12° 06'.

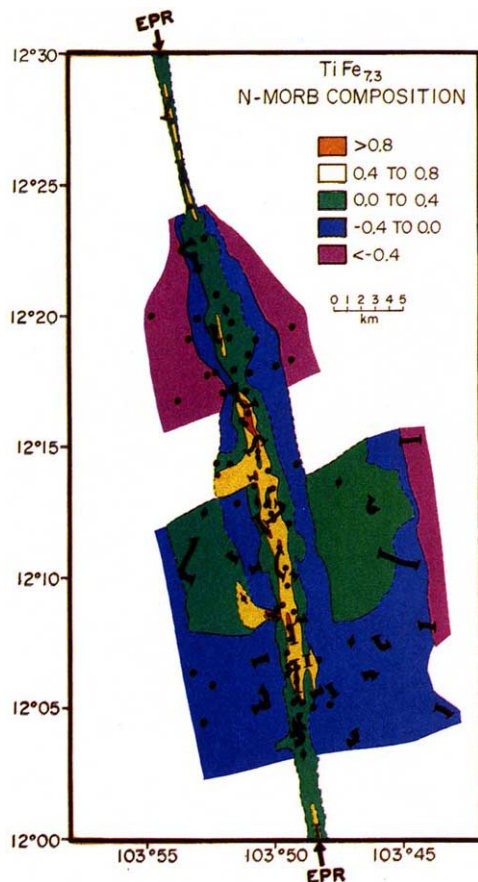


FIG. 3 Compositional map of the $TiFe_{7.3}$ function, for N-MORB samples only. The location of the EPR axis is indicated by arrows at the northern and southern boundaries of the map. Sample locations are shown as circles (rock cores) and lines terminated by bars (dredges). The colour change interval for $TiFe_{7.3}$ is 0.4, which is also the 2σ error. Therefore, $TiFe_{7.3}$ values of samples in one colour interval are within error of $TiFe_{7.3}$ in the next higher or lower interval, but are always significantly different from those which are two intervals away. The colour fields represent compositional regions, analogous to a geological map of different flow units on a subaerial volcano. They do not represent contours of a continuously varying field, as in a bathymetric or magnetic intensity map. Thus even with a progressive change in magma composition through time, the map can contain discontinuities. Adjacent colour fields of very different $TiFe_{7.3}$ intervals, resulting for example from off-axis volcanism, are possible. The data are limited by irregular and sometimes sparse sample coverage, and by a range in $TiFe_{7.3}$ which is only five times the 2σ error. Consequently, the boundaries of the colour fields are drawn with some interpretive licence. The detailed bathymetry²² was used as an aid in interpolating boundaries. In a few cases (indicated by a slash through their symbols), data have not been contoured strictly according to their $TiFe_{7.3}$ but have been included in the next higher or lower interval. The easternmost three dredges are located on a ~200m-high, east-facing scarp, and may have sampled lower stratigraphic levels.

the youngest volcanics, T-MORB, to the central, 19-km deval-bounded segment. This short wavelength of segmentation apparently did not exist ~5,000–40,000 yr ago: there is no evidence for segmentation within the N-MORB compositions along the present-day ridge axis, nor along the axial highs or flanks. At distances of both 1 km and 2 km from the ridge, single controlling liquid lines of descent fit basalt compositions from at least 40 km along strike ($12^{\circ}04' - 12^{\circ}27'$). During an even earlier period (3–8 km), the ridge seems to have had several petrological segments, although the lengths are not well constrained by our sampling.

Modification of this picture of ancient segmentation would be necessary if off-axis volcanism were extensive. Additionally, lava flows originating on-axis could overflow the graben and create young lava plains off-axis. Circumstantial support for these possibilities comes from the smoothed terrain, conical bathymetric forms²³, and relative absence of faults¹¹ in the far eastern side of the $TiFe_{7.3} = 0.0$ to 0.4 region, east of the central ridge axis segment (Fig. 3). This region might reflect excess volcanism covering older, lower- $TiFe_{7.3}$ flows. But a symmetrical petrological region also exists west of the ridge axis, on normal abyssal hill terrain, so we infer that both primarily represent petrological segmentation.

The observed petrological pattern raises the possibility of geochemical stripes of composition in the basalts of the sea floor. These stripes will be irregular because of changes in segmentation that occur during the progressive temporal changes in composition, perhaps with some irregularities resulting from off-axis eruptions. An attempt to synthesize the observations and discussion in this and later sections is presented in Fig. 5. Because the temporal changes in magma chemistry take place over 10^4 years, they provide potentially an order of magnitude finer resolution than the reversals in the magnetic field that

occur at 10^5 to 10^6 years. Geochemical stripes, in combination with bathymetry and better dating techniques, may reveal the history of petrologic and structural segmentation that created the fine-scale fabric of the sea floor.

On- and off-axis eruption of T-MORB?

The T-MORBs inject an element of chaos into the ordered pattern of N-MORB distribution. T-MORB occur throughout the graben of the central segment, so they clearly can occupy the axial magma plumbing system. On the other hand, their isolated occurrences off-axis in the central segment could be caused by sporadic, low-volume, off-axis eruptions (Figs 1 and 5). Supporting evidence comes from their apparently small areal coverage and random distribution, and from their recovery frequently in the same dredge hauls with N-MORB that show regular spatial variations over large distances.

The T-MORB of the central segment seem to occur in small flows, to judge from camera, SeaMARC and sample distribution. Such compositions may always be present beneath the ridge, but ordinarily mix with the preponderant N-MORB magma as they pass through the axial magma system. This may explain the continuum of incompatible trace element compositions observed between N- and T-MORB.

In this scenario, the T-MORB magmas are always present in small volumes, but are observed on the sea floor only under certain conditions. (1) In the early or late stages of a magmatic cycle, T-MORB magmas might be able to erupt without mixing with N-MORB magmas. (2) Isolated eruptions could occur off-axis, where the T-MORB magmas may reach the surface without being greatly diluted by the N-MORB dominated axial magma system. (3) In regional surveys of the EPR, T-MORB have been recovered preferentially at structural offsets^{6,33}, where magmatism is likely to be less robust, magma reservoirs may be

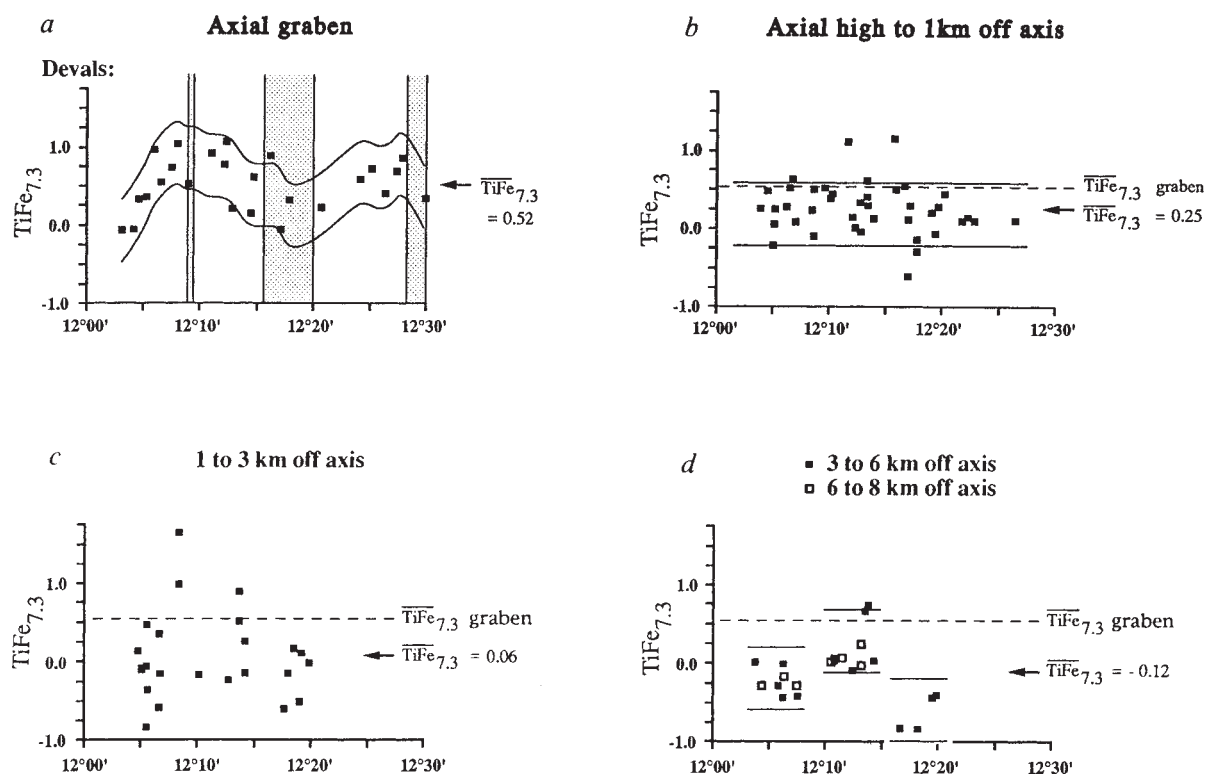
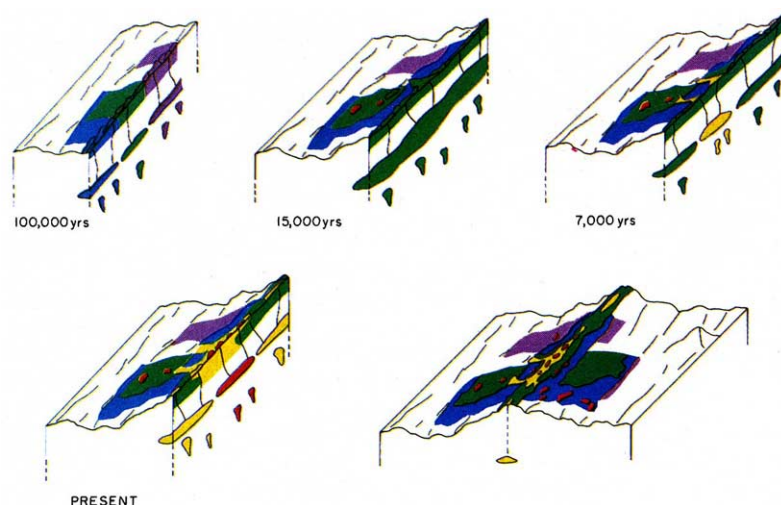


FIG. 4 Comparison of basalt compositions (N-MORB only) between 12° 00' and 12° 30' at successively greater distances from the ridge axis. Only samples from the 1989 cruise are included, as they are better located. Distances are measured along flowlines perpendicular to the ridge axis. In *a*, shading indicates the locations of devals (and thus present-day segmentation) along the ridge axis. These 'zero-age' segment boundaries might not be relevant to other panels if, as the chemical data suggest, the small-scale segmentation has changed with time. Parallel solid lines indicate the ± 0.4 analytical error (2σ); this defines the range in which samples are not significantly different and may lie along similar liquid lines of descent. In *a*, the solid lines enclose the error envelopes following a running average of

2 minutes of latitude. The shaded regions show the present locations of devals, which generally correspond to lower- $\text{TiFe}_{7.3}$ samples. There are variations in $\text{TiFe}_{7.3}$ along strike, with lower- $\text{TiFe}_{7.3}$ samples at 12° 04', between 12° 15' and 12° 20', and possibly at 12° 30'. See discussion in text. In *b*, almost all samples plot within error of a single $\text{TiFe}_{7.3}$ value for the entire length of ridge investigated. *c* shows the largest range in $\text{TiFe}_{7.3}$, with significant variations at given latitudes. In *d*, there seem to be three distinct chemical groups. The mean $\text{TiFe}_{7.3}$ is indicated for the data plotted in each panel. The dashed line shows the mean $\text{TiFe}_{7.3}$ for the graben as a reference, to emphasize the progressive decrease in $\text{TiFe}_{7.3}$ with distance from the graben.

FIG. 5 Model illustration of the temporal evolution of the EPR at 12° 00'–12° 30' N. The colours represent N-MORB with different $\text{TiFe}_{7.3}$ (see Fig. 3), except for red which represents T-MORB. Outlined flows are from active volcanism in each sketch. East–West horizontal exaggeration is $\times 2$, and vertical exaggeration of bathymetry is $\times 30$. The magma chambers are not to scale. The progressive shift of chemistry in the axial magma reservoir with time is based on the observed systematics of basalt composition. The variations in size and continuity of the reservoir reflect possibilities raised in the discussion. In all parts of the figure, magma was probably injected into the reservoirs at multiple locations along the ridge. The first four sketches show snapshots of the axis and the region west of it at different times. At 100,000 yr before present, three disconnected magma chambers are shown erupting petrologically distinct magmas. By 15,000 yr (and perhaps as early as 40,000 yr), the entire length of ridge apparently erupted the same magma type and thus had one petrological segment. Here it is shown as a single, large-volume, continuous crustal magma system, but it might instead have had several disconnected magma bodies with similar magmas. Off-axis magmatism is suggested. At 7,000 yr, the system again has multiple segments. At present, there are three petrological segments bounded by devals. In the 12° N region we see a consistent temporal trend within the N-MORB, defined by the $\text{TiFe}_{7.3}$ systematics; further sampling is needed to determine whether it is part of a repeating pattern and whether the pattern extending further off axis has a sine wave, sawtooth or other shape.



discontinuous or nonexistent, and hence mixing may be much less efficient^{6-10,34}.

It also seems, however, that large volumes of T-MORB can dominate the axial plumbing system. The swollen and shallow ridges north of the Orozco transform and south of the 9° 03' N OSC erupt almost exclusively T-MORB, and provide conclusive evidence for large pulses of T-MORB erupted at the axis^{6,25,35}. The large off-axis T-MORB province in the southeast of our study region thus poses a puzzle. If we have sampled a surface veneer from off-axis volcanism, its large areal extent seems inconsistent with the normal abyssal hill topography in this region. Alternatively, if it resulted from a time when T-MORB dominated the axial system, one would expect a symmetrical counterpart west of the axis, which our limited sampling did not identify.

Small size of magma chambers

Because all eruptions in the axial graben must be very young, the occurrence of adjacent T-MORB and N-MORB within the graben shows that the composition of the axial magma system can change substantially over short time intervals (hundreds to thousands of years). The geological evidence described earlier suggests that T-MORB are the youngest flows. Their small size suggests a small magma pulse, in which case small quantities of magma over short time intervals can change the composition of the axial magma reservoir. This suggests in turn small volumes of resident magma. Even a reservoir several hundred metres thick and a few kilometres wide⁷⁻⁹ has a substantial capacity. These data therefore suggest that the portion of the magma lens at the axis that can be mixed and erupted may be even smaller than was thought^{7-9,34}.

Petrological segments and magma supply

Our data set allows insight into the spacing of melt delivery along the EPR, and the relationship between petrological and structural segmentation. Earlier work demonstrated that multiple supply of magma from the mantle to the crust must occur within a section of ridge bounded by OSCs⁶. The new 12° N data reveal distinct liquid lines of descent for N-MORB domains spaced from 15 to >40 km along strike, as well as T-MORB domains on similar or even smaller length scales. Thus these data confirm the earlier conclusion and show that it applies to distinctions among N-MORB parental magmas as well as to the T-MORB (illustrated in Fig. 5). This finding contrasts with observations for some Hawaiian and Icelandic volcanos³⁶⁻³⁷, where eruptions are commonly controlled by a central feeder system at distances of many tens of kilometres. But such events may occur occasionally on the EPR, and we do see evidence

for a limited time interval ('axial high time interval') of a long section of ridge on a single liquid line of descent.

Changes in the petrological segmentation may correlate with temporal variations in the abundance of magma supply and be used to determine the timescales on which supply variations occur. Abundant magma supply may result in petrological segments that extend over long stretches of ridge, whereas less voluminous magma supply may lead to shorter petrological segments. A change in magma supply may cause a reorganization of the length scale. This inference from the petrological data is intriguingly similar to the picture envisaged by Detrick *et al.*⁷ on the basis of their multichannel seismic data, and is also analogous to a suggestion by Macdonald and Fox³⁸ in reference to OSC-bounded structural segmentation. We should recognize, however, that petrological segments need not correspond to structural segmentation. A large pulse of magma at a single point on the ridge might feed several deval-bounded structural segments by along-strike flow through the crustal obstructions in the magma plumbing system, or multiple pulses with similar N-MORB composition could supply separate, deval-bounded structural segments. In both cases, a single petrological segment would exist over several structural segments.

Concluding remarks

The systematic spatial zonation shown here in basalt composition around a 50-km length of the EPR axis can be seen only when variations of the N-MORB and T-MORB are considered separately. The systematic compositional changes hold promise for elucidating the diverse processes that create the sea floor, and for determining temporal changes in the volumes and patterns of melt delivery from the mantle. The fine resolution of the petrological tool permits inferences that are not possible from magnetics or bathymetry alone.

This widespread petrological mapping of the sea floor was possible only through rock coring from a surface vessel, which allowed better spatial coverage than a submersible, and higher sampling speed and precision than dredging. Petrological mapping must, however, be connected to geological mapping. The combination of these types of data should resolve many questions remaining from the present study. For example, understanding the distribution and extent of off-axis volcanism will require stratigraphic sampling, bottom observations and, ultimately, precise dating. We will need to know flow volumes and their surface dimensions to interpret the compositional maps better and to find the optimal sampling scale. Geological relationships will also be necessary to determine how frequently compositional changes occur within the crustal magma bodies. □

Received 10 April; accepted 4 September 1992.

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ACKNOWLEDGEMENTS. We thank the members of the Venture Leg 2 Scientific Party: P. Banks, A. Barone, J. Charters, D. Chayes, R. Comer, L. Delgado, L. Davidson, G. Eberhart, M. Edwards, A. Howard, E. Humler, A. Martin, T. Plank, W. Smith, M. Thatcher, J. Toth and N. Vaslet. We also thank the officers and crew of the RV *Thomas Washington* for their work, H. Bougault for a large-scale version of the I.F.R.E.M.E.R maps, S. Tighe for customized maps from the JOI Synthesis bathymetric data base, K. Macdonald for a large-scale copy of the SeaMARC II mosaic, E. Humler for assisting with microprobe analyses, and D. Fornari, M. Perfit and R. Kinzler for discussions. This work was supported by the NSF.

Mutations in the channel domain of a neuronal nicotinic receptor convert ion selectivity from cationic to anionic

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Introduction by site-directed mutagenesis of three amino acids from the MII segment of glycine or γ -aminobutyric acid (GABA_A) receptors into the MII segment of $\alpha 7$ nicotinic receptor was sufficient to convert a cation-selective channel into an anion-selective channel gated by acetylcholine. A critical mutation was the insertion of an uncharged residue at the amino-terminal end of MII, stressing the importance of protein geometrical constraints on ion selectivity.

NEUROTRANSMITTER-GATED ion channels such as the nicotinic acetylcholine, glycine, GABA_A or 5-hydroxytryptamine (5HT₃) receptors are transmembrane allosteric proteins made up of homologous subunits pseudo-symmetrically arranged around an axial channel¹⁻⁴. The hydropathy profile of the subunits reveals four potential membrane-spanning hydrophobic segments, named MI to MIV, and convergent photo-affinity labelling⁵⁻⁷ and mutagenesis experiments^{8,9} with nicotinic acetylcholine receptors (nAChRs) point to a contribution of MII to the walls of the ion channel². Chemical kinetics^{10,11} and electrophysiological^{12,13} experiments show that this receptor protein may exist in several interconvertible states (resting, conducting and desensitized) which differ by their ligand-binding and channel-opening properties. In the homooligomeric nAChR composed of the $\alpha 7$ subunit of chick brain^{14,15}, the mutation of a single hydrophobic residue within MII (Leu 247 to Thr) causes noticeable modifications of the response to acetylcholine (ACh), which may represent the conversion of a high-affinity conformation from a closed desensitized state into a conducting state^{16,17}. These results suggest that single amino-acid substitutions may differentially affect the properties of the ion channel in the various conformations of the receptor protein.

The anion-selective¹⁸ glycine and GABA_A receptors^{1,3} display homologies with the cation-selective nAChRs. In particular, in MII (Fig. 1) the following amino acids, which contribute to ion permeation in the nAChR, are conserved: the cytoplasmic ring of negatively charged amino acids (position 234 in $\alpha 7$ receptor⁹), the two polar rings of threonines or serines (position 244 in $\alpha 7$, refs 5, 6, 19, 20, and position 248 in $\alpha 7$, ref. 21) and the hydrophobic ring of leucines (position 247 in $\alpha 7$; refs 7, 16, 17, 22). These sequence homologies and the finding that the glycine receptor (GlyR) MII peptides form ion channels in membranes²³, indicated that MII participates in anion permeation in the glycine and GABA_A receptors^{1,3}. Yet the N-terminal end of MII in all these anionic channels includes an additional amino acid located in the short segment linking MI to MII (between positions 236 and 237 in $\alpha 7$), which could contribute to the difference of ion selectivity between ACh and GABA_A/glycine receptors.

Here we show that combined substitutions and/or addition of amino acids within (or near) the MII segment from the nicotinic $\alpha 7$ receptor with homologous residues from the GlyR $\alpha 1$ subunit²⁴ suffice to convert $\alpha 7$ ion-channel selectivity from cationic to anionic. Also, some of the amino-acid permutations alter monovalent versus divalent cation selectivity, as similarly

described for glutamate receptors²⁵⁻²⁷. Yet in addition to the alteration in ion selectivity, important changes occur in agonist/competitive antagonist sensitivity and desensitization properties of the response to ACh.

Neuronal $\alpha 7$ channel is permeable to cations

Several neuronal nAChRs are permeable to calcium²⁸⁻³⁰. When these receptors are expressed in *Xenopus* oocytes, the calcium flux through their channel leads to activation of a Ca²⁺-dependent chloride conductance²⁹⁻³¹, sensitive to calcium chelators³². This chloride current was used here to detect calcium permeability through nAChRs. Therefore, the chloride contribution to ACh-evoked ionic responses was determined, at various potentials, as the difference between currents measured before and after injection of the calcium chelator BAPTA³³ in oocytes expressing $\alpha 7$ wild-type (WT) or mutant receptors.

When expressed in *Xenopus* oocytes, $\alpha 7$ WT receptors elicited large ACh-evoked inward currents reversing at -15.4 ± 4 mV (Fig. 2a). Substitution of 90% external chloride by the large anion isethionate shifted the reversal potential close to 0 mV ($E_{rev} = 1.8 \pm 3.7$ mV; Table 1), providing evidence for a contribution of chloride ions to the current. After injection of BAPTA, ACh-activated currents reversed at a potential (3.8 ± 4.8 mV, Table 1) similar to that determined on substitution of external chloride, indicating that the current carried by chloride ions, which represented up to 50% of the ionic response at -40 mV (Fig. 2b), had been significantly reduced by chelation of internal calcium. Indeed, at -15 mV, the slow onset of chloride influx (outward current), which follows an inward cationic current, was no longer observed after injection of BAPTA (Fig. 2d). These data thus support the conclusion that $\alpha 7$ WT receptor, like other neuronal nAChRs²⁸⁻³⁰, is selective for cations and permeable to calcium.

Conversion from cationic to anionic selectivity

In a first attempt to convert ion-channel selectivity, we constructed chimaeric complementary DNAs encoding nicotinic $\alpha 7$ receptors in which the segment linking MI to MII and also half or all of the MII segment (from amino acid $\alpha 7$ -234 to 245 or from 234 to 258) was exchanged with that of the glycine $\alpha 1$ subunit. No detectable current in response to ACh application was recorded in oocytes injected with these chimaeric cDNAs. In a second step, instead of substitution in a block, amino acids presumed to face the lumen of the ion channel in the GlyR, assuming that MII is organized as an α -helix^{2,6-9,21,22,34,35}, were introduced into $\alpha 7$ nAChR.

TABLE 1 Sequence and properties of the MII mutants

Type	Sequence	Amplitude μ A Mean value	EC ₅₀ ACh	nH	EC ₅₀ DH β E	nH	Control	Reversal potential mV Cont/BAPTA	Iset	Iset/BAPTA	Selectivity Cationic/ Anionic
α 7-WT	DSG-EKISLGITVLLSLT VFM L LVAE	0.63 (n=98)	115.00	1.4	C.A	—	-15.4 $\pm$ 4.0	3.8 $\pm$ 4.8	1.8 $\pm$ 3.7	ND	C
α 7-1	---PA---G-----T---SG---N	0.21 (n=75)	0.24	1.6	0.30	1.4	-19.0 $\pm$ 4.6 -23.2 $\pm$ 3.4 -22.4 $\pm$ 3.1	-19.0 $\pm$ 4.9 -18.6 $\pm$ 4.8	27.0 $\pm$ 7.6 24.3 $\pm$ 8.6 24.4 $\pm$ 9.8	29.3 $\pm$ 3.0 (a) 25.2 $\pm$ 6.6 (b) (c)	A
α 7-2	---PA-----T-----	0.13 (n=23)	0.23	1.8	0.94	1.4	-25.8 $\pm$ 5.4	ND	15.3 $\pm$ 8.8	12.5 $\pm$ 3.0	A
α 7-3	---PA-----	—	—	—	—	—	—	—	—	—	—
α 7-4	---T-----	5.50 (n=38)	0.63	2.0	9.27	1.4	-17.7 $\pm$ 6.6	-4.2 $\pm$ 5.6	36.3 $\pm$ 7.5	2.2 $\pm$ 5.7	C
α 7-5	---A-----	0.13 (n=23)	194.00	1.4	ND	ND	-14.2 $\pm$ 2.1	-13.5 $\pm$ 1.7	ND	ND	C
α 7-6	---A-----T-----	2.90 (n=10)	0.98	2.0	3.69	1.4	-18.5 $\pm$ 1.3	-18.5 $\pm$ 3.9	-18.5 $\pm$ 1.3	-19.0 $\pm$ 3.4	C
α 7-7	---P-----	0.02 (n=10)	ND	ND	ND	ND	ND	ND	ND	ND	ND
α 7-8	---A---G-----T---SG---N	4.27 (n=23)	5.45	2.8	ND	ND	-10.9 $\pm$ 2.8	-12.2 $\pm$ 2.9	-11.5 $\pm$ 3.8	ND	C
α 7-9	---AA---G-----T---SG---N	0.09 (n=09)	ND	ND	ND	ND	-18.3 $\pm$ 3.4	-20.3 $\pm$ 3.5	12.6 $\pm$ 1.1	29.5 $\pm$ 9.5	A

The complete sequence of the MII segment is given for α 7 WT. For α 7-1 to α 7-9 mutant sequences, only those residues differing from the WT sequence are shown. The gap between positions 236 and 237 of the WT is indicated when present (mutants α 7-4, α 7-5, α 7-6 and α 7-8). Mean whole-cell responses were measured at 100 μ M ACh. EC₅₀ values for ACh and DH β E are given in μ M. Reversal potential of α 7-1 mutant was determined after (a) 0.6 s 100 μ M ACh application, (b) 0.6 s 0.2 μ M ACh application and (c) 3.6 s 100 μ M ACh application; ACh pulses lasted 2 s (a, b) or 4 s (c). Similar reversal potentials were determined at 1 μ M and 100 μ M ACh for mutants α 7-2, α 7-4 and α 7-6, and at 5 μ M and 100 μ M for mutant α 7-8. No ionic response could be recorded in oocytes injected with α 7-3 cDNA ND, not determined; CA, competitive antagonist; IC₅₀ of DH β E for WT receptor, 1.6 μ M (ref. 36). Similar recordings were obtained for every mutant in at least 5 cells from more than one donor. **Mutagenesis:** Mutants were prepared as described in ref. 16. Their complete coding sequence were systematically checked. Half- or full-length chimaeras of the MII segment were prepared by the same method.

Electrophysiology: Oocytes were prepared, injected and recorded as described⁵¹. Recordings were typically made two or three days after injection. For voltage-clamp measurements, cells were incubated in OR2 medium (NaCl, 82.5 mM; KCl, 2.5 mM; CaCl₂, 2.5 mM; MgCl₂, 1 mM; atropine, 0.5 μ M; HEPES, 5 mM, pH 7.4) and challenged by ACh application. Solution changes were complete within 100 ms. Data from one oocyte are given in the figures and mean values $\pm$ s.e.m. determined from 5–10 oocytes are given in Table 1. Dose-response curves were obtained by plotting the peak of the ACh-evoked currents, measured for 2-s applications, as a function of the logarithm of the agonist concentration. Cells were held at -100 mV. Mean currents obtained in five cells expressing each mutant were normalized to the values determined at saturating agonist concentration. The empirical Hill equation, adjusted by weighted least-square algorithm, yielded the apparent 50% effective concentrations (EC₅₀) and Hill coefficients (nH) given in Table 1. No residual currents were measured for α 7 WT and α 7-1 receptors after 30-min exposure to 100 nM α -bungarotoxin. *I-V* curves were obtained in the voltage range between -50 and +40 mV and generated by subtracting passive membrane currents from currents evoked in the presence of ACh at each potential. For ion selectivity experiments, chloride concentration was decreased from 92 mM to 9.5 mM by replacing NaCl by Na-isethionate (experiments denoted isethionate), sodium concentration was decreased from 82.5 mM to 41.25 or 8.25 mM by replacing 50% or 100% of NaCl by mannitol to preserve osmolality (experiments denoted 1/2Na-mannitol or mannitol). To minimize changes in junctional potential on ionic substitution, an agar-bridge was used as a ground electrode. Residual potential changes were estimated and corrected for. **BAPTA injection:** Control responses were first recorded and a third electrode, similar to those used for cDNA injection was poked into the oocyte. This electrode was made with a calibrated capillary and filled with a solution containing 0.1 M BAPTA in NaCl, 82.5 mM; KCl, 2.5 mM; HEPES, 5 mM, pH 7.4. Small positive pressure pulses, calibrated to produce 10 nl injections were used to inject BAPTA. Four pulses were normally applied for standard injections. The 40 nl BAPTA at 0.1 M must roughly correspond to 7 mM internal concentration of chelating agent. The injection was monitored under visual control and effectiveness of BAPTA action was checked electrophysiologically. Injection of larger volumes never showed any significant differences with standard procedure. **Single-channel recordings:** Quality and response of oocytes used for single-channel recordings were first assessed with the dual electrode voltage clamp and vitelline membrane was then removed as described¹⁶. Because of apparent clustering of the channels in the oocyte membrane, AChRs were first localized using a puffing electrode and whole-cell recording. Pipettes used for outside-out patch recordings were sylgarded to reduce capacitance and contained (in mM) KF, 70; EGTA, 20; HEPES, 5; pH 7.4. The bath solution contained OR2 in control experiments and OR2 0.5 Na⁺/0.5 mannitol in ionic substitution experiments. Ground electrode consisted of an agar bridge to minimize changes in junction potential on substitution of Cl⁻ in the bath. Data were filtered at 1.5 kHz and sampled at 5 kHz.

The first mutant investigated (α 7-1, Table 1) included one additional amino acid (a proline residue) located between α 7-G236 and α 7-E237 at a position now referred to as 236' and six additional point mutations at positions 237 (Glu $\rightarrow$ Ala), 240 (Ser $\rightarrow$ Gly), 251 (Val $\rightarrow$ Thr), 254 (Leu $\rightarrow$ Ser), 255 (Leu $\rightarrow$ Gly) and 258 (Glu $\rightarrow$ Asn).

Expression of α 7-1 mutant in *Xenopus* oocytes yielded functional ACh-gated ion channels. The quaternary structure of the WT and mutant receptors was not controlled in this expression system, but there were no major differences in Hill coefficient (Table 1) or sensitivity to α -bungarotoxin (not shown) between α 7 WT and α 7-1, indicating that the overall structure of the protein is probably not much altered. But several other properties of this mutant greatly changed, as in the case of the previously described L247T mutant^{16,17}. The whole-cell current-voltage relationship became linear (Fig. 3a), the dose-response curve for ACh was shifted by 500 times towards lower concentrations (Table 1), and most of the desensitization of the response to ACh was abolished (Fig. 3b, compared with WT responses in Fig. 2c). Thus, in α 7-1, as in L247T (ref. 16), a new functional high-affinity (effector concentration for half-maximal response, EC₅₀ = 0.24 μ M) open state exists, which could correspond to a desensitized but conducting 'D*' state of the nAChR¹⁶. Consistent with this notion, we noticed that the onset of permeability

changes of mutant α 7-1 was slow compared with WT (Fig. 3b) and that dihydro- β -erythroidine (DH β E) behaved as an agonist (Table 1). This is in contrast with the WT receptor which displays a single low-affinity (EC₅₀ = 115 μ M) conducting state, referred to as the 'active' (A) state, which rapidly desensitizes and is competitively inhibited by DH β E³⁶.

Ionic currents obtained from whole-cell recordings reversed at -19.0 $\pm$ 4.6 mV. Substitution of 90% external chloride ions by isethionate (gluconate or methane sulphonate, not shown), shifted the reversal potential of ACh-activated currents towards positive voltages (E_{rev} = 27 $\pm$ 7.6 mV) independently of the concentration of ACh or the duration of agonist application (Table 1). These shifts were well described by the Goldman-Hodgkin-Katz relationship (Fig. 3d) for chloride-specific channels, indicating that the ACh-activated currents were almost entirely carried by chloride ions. Furthermore, reversal potential (as the kinetic properties of the response) did not change in the presence of internal BAPTA (E_{rev} = 29.3 $\pm$ 3 mV, Fig. 3a), excluding a significant contribution of calcium-activated chloride channels to the ionic response of the oocyte. Replacement of 100% extracellular sodium chloride by mannitol (Fig. 3c) shifted the reversal potential towards positive voltages (E_{rev} = 43.6 $\pm$ 6.6 mV, n=8; δE_{rev} = 62 mV) similar to those determined with substitution of chloride by isethionate, indicating that

FIG. 1 Comparison of MII sequences from subunits of the cation-selective nicotinic α 7 receptor with those of anion-selective glycine α 1 (ref. 24) glycine β (ref. 49), GABA_A α 1 and β 1 (ref. 38) receptor subunits. Numbers refer to α 7 sequence. Amino acids previously mutated in nAChRs (see text) and conserved in glycine and GABA_A receptors are indicated by stars. Amino acids mutated in this study are shown in circles (insertion between 236 and 237 and substitutions at positions 237, 240, 251, 254, 255 and 258).

		MII																									
		234	236	237	240											251	254	255	258								
AChR	α 7	D	S	G	(E)	K	I	(S)	L	G	I	T	V	L	L	S	L	T	(V)	F	M	(L)	(L)	V	A	(E)	
GlyR	α 1	D	A	A	P	A	R	V	G	L	G	I	T	T	V	L	T	M	T	T	Q	S	S	G	S	R	A
GlyR	β	D	A	S	A	A	R	V	P	L	G	I	F	S	V	L	S	L	A	S	E	C	T	T	L	A	A
GABAR	α 1	E	S	V	P	A	R	T	V	F	G	V	T	T	V	L	T	M	T	T	L	S	I	S	A	R	N
GABAR	β 1	D	A	S	A	A	R	V	A	L	G	I	T	T	V	L	T	M	T	T	I	S	T	H	L	R	E
		*			*				*		*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*	*

permeability to sodium was not significant ($P_{Na}/P_{Cl} < 0.05$). As a probable consequence of the low amplitude of whole-cell currents (210 nA, Table 1), no single-channel recordings could be obtained (17 oocytes from 7 donors; 122 patches tested) to characterize further $\alpha 7-1$. Yet the available data support the conclusion that, in the presence of ACh, the current flows through a high-affinity D^* state of the $\alpha 7-1$ channel and is mostly carried by chloride ions.

To identify further the critical residues involved in the conversion of ion selectivity from cationic ($\alpha 7$ WT) to anionic ($\alpha 7-1$), the number of amino acids exchanged was progressively reduced. The mutant $\alpha 7-2$ contained a proline at position 236' and amino acids 237 and 251 were occupied by Ala and Thr, respectively. As for $\alpha 7-1$, this mutant formed functional channels of high apparent affinity for ACh, that can be activated by DH β E (Table 1) and that display weakly desensitizing (Fig. 3f), and non-rectifying (Fig. 3e) ionic responses. Also, substitution of external anions in the presence or absence of internal BAPTA yielded results similar to those of $\alpha 7-1$ (Fig. 3e, Table 1); thus $\alpha 7-2$ would, again, conduct ions in the D^* state and be selective for anions.

Further reduction in the number of point mutations (mutants $\alpha 7-3$ to $\alpha 7-5$) did not invert the sign of ion selectivity of $\alpha 7$ WT. To understand how three amino-acid substitutions (236' $\rightarrow$ Pro, Glu 237 $\rightarrow$ Ala and Val 251 $\rightarrow$ Thr) gave the multiple effects observed in $\alpha 7-1$ and $\alpha 7-2$, we analysed the properties of each mutation separately.

Desensitization but not selectivity altered in $\alpha 7-4$

As shown in Fig. 4 and Table 1, this single Val 251 $\rightarrow$ Thr point mutation (mutant $\alpha 7-4$) accounted for the changes in apparent affinity for ACh, sensitivity to DH β E (Table 1), response desensitization and current rectification observed with the multiple mutants $\alpha 7-1$ and $\alpha 7-2$.

Single-channel recordings from outside-out patches (Fig. 4c) further indicated that mutant $\alpha 7-4$, as the L247T mutant¹⁶,

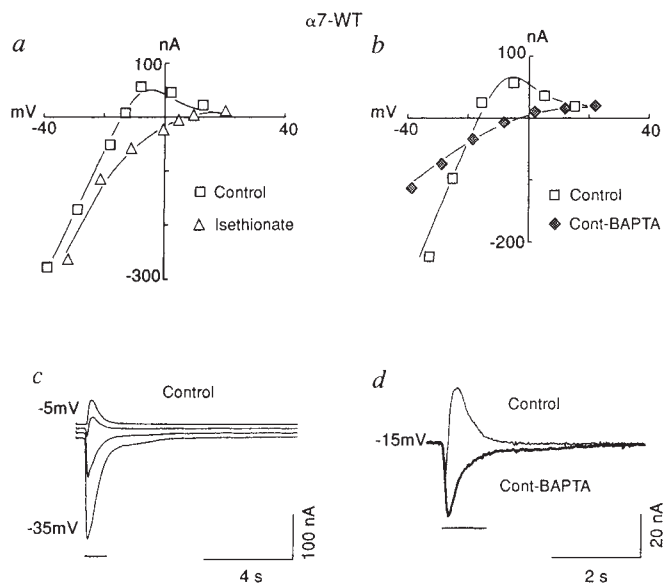


FIG. 2 *a, b*, Current-voltage ($I-V$) relationships of $\alpha 7$ WT receptor. Peak currents evoked by 1-s exposure to 100 μ M ACh were measured at several holding potentials, first in control conditions (Control) in OR2 medium (see Table 1 legend), then after substitution of 90% external chloride by isethionate (isethionate), or ≥ 2 min after injection of 4 nmol of the Ca^{2+} -chelating agent BAPTA (Cont-BAPTA). *c*, Time course of the ACh-evoked currents recorded under control conditions at four holding potentials separated by 10 mV increments. *d*, Currents recorded at -15 mV, before (Control), or after BAPTA injection (Cont-BAPTA). Further injection of BAPTA did not induce other detectable changes in the ACh-evoked current.

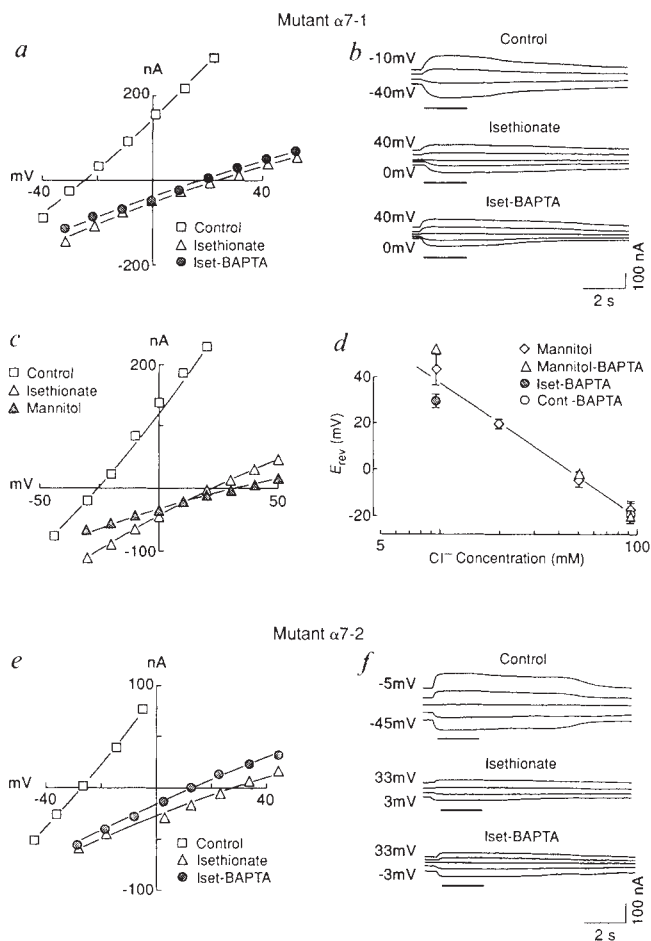


FIG. 3 *a, I-V* relationship of the $\alpha 7-1$ mutant receptor. The $I-V$ curve was determined first in control condition (Control) with 2-s ACh applications. Ninety per cent of chloride ions of the perfusion medium were then replaced by isethionate and the $I-V$ curve determined again (Isethionate). Injection of BAPTA did not induce significant changes in this $I-V$ relationship (Iset-BAPTA). Lines through the data points correspond to the Goldman-Hodgkin-Katz (GHK, ref. 50) equation adjusted by weighted least-square algorithm assuming a permeability of 5% for isethionate relative to chloride. The parameters used correspond, respectively, to: permeability, internal and external ion concentration (in mM). Control: 0.27, 34, 92; Isethionate: 0.28, 34, 9.5, for Cl^- and 0.013, 0, 82.5 for isethionate; Iset-BAPTA: 0.24, 34, 9.5, for Cl^- and 0.022, 0, 82.5 for isethionate. *b*, Time course of the ACh-evoked currents recorded in control, isethionate and isethionate-BAPTA conditions. Traces were recorded at 10 mV increments. Horizontal bars correspond to ACh applications (100 μ M). *c*, $I-V$ curve determined with 2-s ACh applications in control condition (control), after substitution of 90% chloride by isethionate (Isethionate), and after substitution of 100% sodium chloride by mannitol (Mannitol). Adjusted parameters for the GHK fit are: Control: 0.22, 33, 92; Isethionate: 0.24, 33, 9.5 for Cl^- and 0.012, 0, 82.5 for isethionate; Mannitol: 0.15, 33, 9.5. *d*, Reversal potential values as a function of the logarithm of external chloride concentration. $I-V$ curves were determined in the presence of 92, 50.5, 19.75 and 9.5 mM external chloride, after substitution of NaCl by mannitol (diamonds). Other reversal potentials, determined after injection of BAPTA, are: triangles: 9.5, 50.5 and 92 mM external chloride (substitution by mannitol); filled circle: 9.5 mM chloride (substitution by isethionate); open circle: 92 mM chloride. Mean $\pm$ s.e.m. reversal potential values were determined in $n=5$ cells. The solid line corresponds to the theoretical Nernst relation. *e*, $I-V$ relationship of the $\alpha 7-2$ mutant receptor obtained using the protocol in *a*. Adjusted parameters for the GHK equation are: Control: 0.16, 32, 92; Isethionate: 0.14, 32, 9.5 for chloride and 0.002, 0, 82.5 for isethionate; Iset-BAPTA: 0.14, 32, 9.5 for chloride and 0.015, 0, 82.5 for isethionate. *f*, Time course of ACh-evoked currents recorded at several holding potentials (10 mV increments) in the three conditions of *e*. Horizontal bars correspond to ACh applications (100 μ M).

exhibited multiple conducting states, one of 54 ± 3 pS ($n=6$) comparable to that of the WT A state, and one conductance of 86.3 ± 5.4 pS ($n=11$), plausibly corresponding to a conducting D^* state. In agreement with this hypothesis, we noticed that DH β E behaved as an agonist of $\alpha 7$ -4 mutant (Table 1). Also after injection of BAPTA, ionic responses did not desensitize and their onset resembled the slow ones observed for $\alpha 7$ -1.

Current reversal potential of mutant $\alpha 7$ -4 ionic responses shifted by 54 mV towards positive voltages on substitution of 90% external chloride (Fig. 4a; Table 1), indicating that an important part of the observed current was carried by chloride ions. But in contrast to $\alpha 7$ -1 or $\alpha 7$ -2 receptor ionic responses, after injection of BAPTA, external chloride substitution produced no effect (from $E_{rev} = -4.2 \pm 5.6$ to $E_{rev} = 2.2 \pm 5.7$ mV, Table 1). Similar results were obtained when Ca^{2+} was substituted by Ba^{2+} ($E_{rev} = -3$ mV, data not shown). As for the $\alpha 7$ WT receptor, these data provide evidence for the activation of calcium-dependent chloride channels. Single-channel current-voltage relationships (Fig. 4c) indicated that reversal potentials of both conducting states were insensitive to changes in external chloride concentrations (not shown) but were shifted towards negative voltages ($\delta E_{rev} = 23$ mV) on twofold reduction of external sodium (see legend to Fig. 4c), consistent with a cation-selective permeability.

Thus two functional features of the channel can be dissociated by mutagenesis: its ability to conduct ions in the D^* and/or A states and its ionic selectivity.

Divalent/monovalent cation selectivity

Glutamate residues at position 237 in $\alpha 7$ WT receptor form the 'intermediate' ring of negatively charged amino acids previously investigated in *Torpedo californica* receptor^{9,20,37}.

In this study with homo-oligomeric $\alpha 7$ neuronal receptor, mutation of Glu237 to Ala removed the whole set of charges from this ring (mutant $\alpha 7$ -5). The receptor still responded to ACh (Fig. 5a) with activation by high agonist concentrations ($EC_{50} = 194 \mu M$) and rapid desensitization of ionic responses in the presence of physiological concentrations of divalent cations (Fig. 5b). These properties are similar to those of the WT receptor which exhibits a low-affinity A conducting state.

In contrast to WT, current-voltage relationships were now linear (Fig. 5a) and reversed at -14.2 ± 2.1 mV. Also, injection of BAPTA in the oocytes, did not affect the reversal potential ($E_{rev} = -13.5 \pm 1.7$ mV) of ACh-evoked currents, suggesting, as proposed for nAChR mutants⁹ and glutamate receptor variants or mutants²⁵⁻²⁷, that this channel is permeable to cations, but has lost its ability to conduct divalent cations (D.B. *et al.*, manuscript in preparation).

Ion selectivity in the same DH β E activated state

As seen on the sequence alignments in Fig. 1, one additional residue, a Pro or an Ala is present between positions 236 and 237 (position 236') at the N-terminal end of MII in the anion-selective Gly and GABA_A receptor channels^{24,38}.

Deletion of this amino acid (a proline) in the chloride-selective $\alpha 7$ -2 receptor resulted in a functional ACh-gated channel (mutant $\alpha 7$ -6). Mutant $\alpha 7$ -6 ionic responses were activated by ACh and DH β E with EC_{50} (0.98 μM and 3.69 μM , respectively) close to those found for mutant $\alpha 7$ -2 (see Table 1), and did not desensitize either in the presence or absence of BAPTA (Fig. 5d). Moreover, their onset was slow and no residual current persisted on removal of the agonist. Altogether, these observations indicate that $\alpha 7$ -6 mutant conducts ions in the same state as $\alpha 7$ -2 mutant, referred to as the D^* conducting state.

Current-voltage relationships were linear. The reversal potential was not changed on injection of BAPTA ($\delta E_{rev} = 0$ mV, Fig. 5c) suggesting that Ca^{2+} does not permeate, and was no longer affected by external chloride substitution ($\delta E_{rev} = 0$ mV), thus indicating that chloride contribution to the current was negligible. Furthermore, whole-cell current reversal potential shifted

from -18.5 ± 1.3 mV to -27.3 ± 1.5 mV ($n=4$) on substitution of 50% external sodium chloride by mannitol (Fig. 5e) as expected for cation-permeable channels, and thus establishing that mutant $\alpha 7$ -6 is now cation-selective and that conversion of ion selectivity can be observed for one given conducting state.

Similar results with regard to cation versus anion selectivity were obtained when deletion of proline 236' was done in the more complex $\alpha 7$ -1 mutant to yield $\alpha 7$ -8 (see Table 1). Conversely, insertion in the cation-selective $\alpha 7$ -8 mutant of either a proline ($\alpha 7$ -1) or an alanine ($\alpha 7$ -9) at position 236' reversed the ion selectivity to anionic, thus suggesting that the length of the segment spacing MI and MII is more critical than the actual nature of the residue inserted.

No analysable (less than 20 nA) ionic currents could be recorded from oocytes injected with mutants $\alpha 7$ -3 and $\alpha 7$ -7

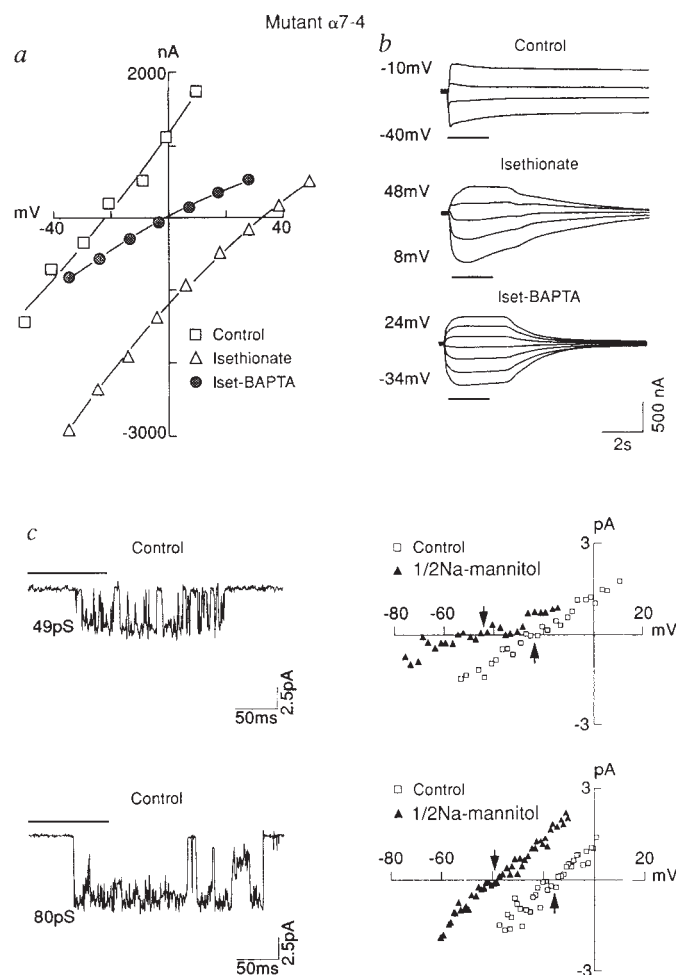


FIG. 4 a, I - V relationship of the $\alpha 7$ -4 mutant receptor obtained using the protocol in Fig. 3a. b, Time course of ACh-evoked currents were recorded at several holding potentials (10 mV increments) in the three conditions of a. Horizontal bars correspond to ACh applications (100 μM). c, Single-channel recordings of $\alpha 7$ -4 mutant receptor. Excised outside-out patches from oocytes expressing mutant receptors were recorded in OR2 medium. Receptor channels were activated by 100-ms puffs of 20 μM ACh (bars). Mutant $\alpha 7$ -4 exhibits multiple conductance levels, illustrated here by a 49 pS (top) channel and an 80 pS (bottom) channel. Recordings are from two different patches at a membrane potential of -100 mV. Current-voltage relationships were constructed using a voltage ramp protocol. Leak currents obtained in the absence of ACh were subtracted from traces showing channel activity. The reversal potential of both channel types (indicated by arrows) shifted from -21.9 ± 2.6 mV ($n=5$) to -45.5 ± 7 mV ($n=3$) for the 49 pS conductance and from -19.6 ± 3.5 mV ($n=4$) to -42.5 mV ($n=2$) for the 80 pS on substitution of 50% of the external NaCl with mannitol.

(Table 1) containing the additional Pro residue, but not the Val251 → Thr mutation.

Discussion

The noticeable sequence homologies between nicotinic, serotonergic 5HT₃, GABA_A and GlyRs (refs 1, 4), suggest that the different properties of these receptors rely on a limited number of key amino-acid side chains within a common polypeptide backbone. For instance, the fact that invertebrates express chloride channels gated by ACh^{38–41} and possibly by glutamate⁴², illustrates the absence of strict relation between neurotransmitter specificity and ion selectivity. Moreover, several chloride channels let cations permeate together with anions (reviewed in ref. 43), indicating that intermediate cation versus anion selectivity may exist and that anion and cation channels may share common structural properties.

Consistent with these observations, we show that introducing appropriate amino-acid residues from the putative channel

domain of a chloride-selective GlyR α 1 (ref. 24) into that of a cation-selective α 7 nAChR^{14,15} allowed the design of an ACh-gated channel now selective for chloride. Mutations of only those residues presumed to face the lumen of the channel yielded functional receptors, whereas exchange of the entire MII segment of the nicotinic α 7 receptor with that of the glycine α 1 receptor did not give functional channels. Possibly this failure resulted from perturbations of the tertiary or quaternary structure of the receptor associated with amino acids located in the non-luminal face of MII segment⁴⁴. In contrast, the success of the individual amino-acid exchange method indicates that the overall geometry of these oligomers is highly similar.

Ionic selectivity was converted after a small number of amino-acid additions and permutations within α 7 sequence: the addition of a Pro (or Ala) residue at the N-terminal end of the MII segment (between positions 236 and 237) and two amino-acid substitutions at positions 237 (Glu → Ala) and 251 (Val → Thr). Unexpectedly, mutations of two rings of negatively charged amino acids (outer (position 258) and intermediate (position 237) rings⁹) to neutral amino acids led to functional receptors (α 7-5, α 7-6, α 7-8) which are still selective for cations. Yet, unlike the WT receptor, those channels mutated at the intermediate ring exhibited low calcium permeability. Thus, although these charged amino acids, in particular from the intermediate ring, might contribute to the selectivity among cations, they do not seem to play a critical role in anion versus cation selectivity.

The most critical mutation for inverting the sign of ion selectivity was the insertion or deletion of a neutral residue modifying the length of the short segment linking MI to MII. This effect is strongly dependent on the nature of the residue at position 251. Indeed, when position 251 is occupied by a valine residue, the receptor displays only a low-affinity conducting state and the insertion at position 236' generally does not yield functional receptors (mutants α 7-3 and α 7-7). When position 251 is occupied by a Thr residue, the receptor displays a high-affinity conducting state but is still selective for cations. The insertion of a neutral residue then converts the ion selectivity from cationic (mutant α 7-6) to anionic (mutant α 7-2). Under these conditions, both anion- and cation-selective channels belong to a high-affinity 'desensitized but conducting' D* state^{16,17} of the receptor.

Proposal of a detailed structural model for cationic versus anionic selectivity of the ion path is premature at this stage. Yet it is tempting to speculate that the conversion of ion selectivity observed on Pro or Ala insertion in mutant α 7-6 and α 7-8 (yielding α 7-2, α 7-1 and α 7-9, respectively), is not directly related to a defined chemical modification within the channel lumen but, rather, to slight changes of the geometry of the MII segments which delineate the channel. Such change would then modulate the accessibility of permeant ions to putative selectivity filters located within or outside MII.

The slow conformational transitions which take place in the course of desensitization of the *Torpedo* receptor protein alter altogether the relative orientation of the subunits⁴⁵ and the structure of sites located at their interfaces^{46,47}. Possibly such dynamic changes in the geometry of the quaternary structure constrain (or even regulate) the selectivity of the channel, as long as the ion path is open in the various allosteric states of the receptor. Consistent with this view is the striking difference of channel properties between the two conducting states of L247T (refs 16, 17) or V251T (α 7-4) (ref. 52) mutants, as well as the difference of size or state of hydration between cations and anions permeating through the various mutant channels. Also, in the case of GABA_A receptors⁴⁸, fast and slow transitions between channel states with different properties have been recorded and display analogies with some of those reported here. Our data further suggest that, as in the case of typical allosteric proteins, the geometry of the quaternary structure of the receptor protein and its ability to undergo conformational transitions might be important parameters for determining the ion selectivity of the ion channel it contains. □

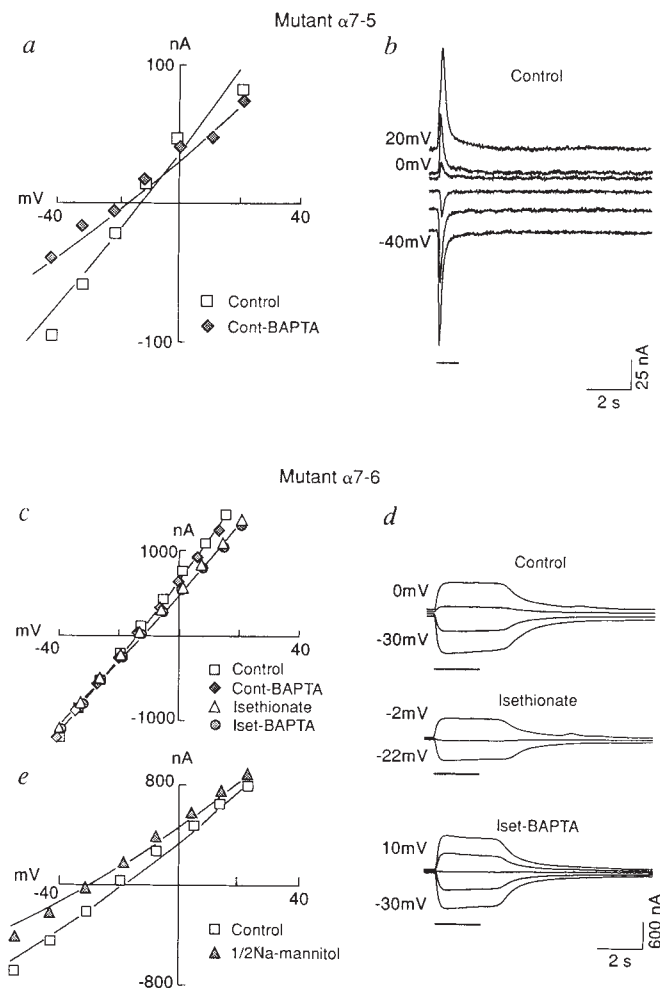


FIG. 5 *a*, *I*-*V* relationship of α 7-5 mutant receptor. Same protocol as in Fig. 2*b* with 1-s exposure to ACh. Adjusted parameters for the GHK equation are: Control: 0.07, 1.9, 82.5 for Na⁺ and 0.11, 89, 2.5 for K⁺; Cont-BAPTA: 0.04, 1.5, 82.5 for Na⁺ and 0.075, 89, 2.5 for K⁺. *b*, Time course of the ACh-evoked currents at several holding potentials (10 mV increments). *c*, *I*-*V* relationship of the α 7-6 mutant receptor. Same protocol as in Fig. 3*a*. Adjusted parameters for the GHK equation are: 1.07, 2.5, 82.5 for Na⁺ and 1.96, 89, 2.5 for K⁺. *d*, Time course of ACh-evoked currents of α 7-6 mutant receptor recorded at several holding potentials in control isethionate and isethionate-BAPTA condition. *e*, *I*-*V* relationships of α 7-6 mutant were determined first in control condition (Control) and after substitution of 50% of NaCl by the mannitol (Na-Mannitol). Adjusted parameters for the GHK fit are: 0.4, 1.8, 82.5 for Na⁺ and 0.78, 89, 2.5 for K⁺ in control conditions and became 0.55, 1.8, 41.25 for Na⁺ and 0.78, 89, 2.5 for K⁺. Horizontal bars represent the ACh applications (100 μ M).

Received 13 April; accepted 3 September 1992.

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ACKNOWLEDGEMENTS. We thank R. Miles, C. Mülle and C. Léna for discussions and reading the manuscript. This work was supported by grants from the Association Française contre les Myopathies, the Collège de France, the Centre National de la Recherche Scientifique, the Ministère de la Recherche, the Direction des Recherches Etudes et Techniques and the Swiss National Science Foundation (to D.B.).

Crystal structure at 1.7 Å of the bovine papillomavirus-1 E2 DNA-binding domain bound to its DNA target

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The dominant transcriptional regulator of the papillomaviruses, E2, binds to its specific DNA target through a previously unobserved dimeric antiparallel β -barrel. The DNA is severely but smoothly bent over the barrel by the interaction of successive major grooves with a pair of symmetrically disposed α -helices. The specific interface is an 'interwoven' network of interactions where the identifying base pairs of the target contact more than one amino-acid side chain and the discriminating amino acids interact with more than one base pair.

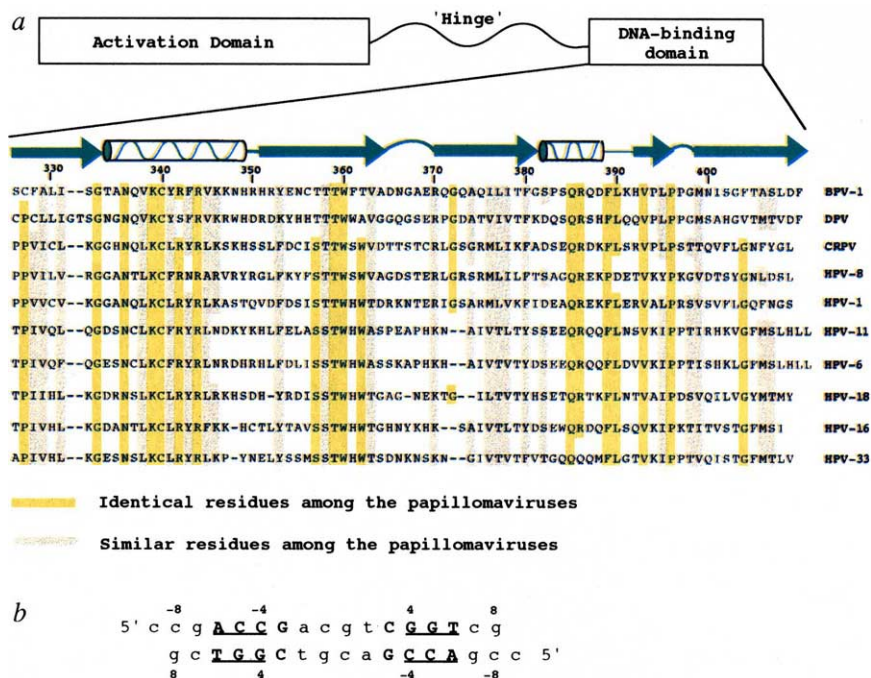
THE papillomaviruses are a family of small DNA viruses that cause hyperplastic epithelial lesions. The most notable pathogens are the human papillomavirus strains HPV-16 and 18, which have been implicated in cervical carcinomas¹, although it is bovine papillomavirus-1 (BPV-1) that has been the most extensively studied (for reviews, see refs 2 and 3). The products of the E2 gene, the proteins E2, E2-TR and E8/E2, are pivotal to the viral life cycle because they regulate viral transcription from all viral promoters and are essential for viral replication^{4,5}. The full-length (410 amino acids) E2 gene product is a *trans*-acting transcriptional regulator. It is also required, in conjunction with the E1 protein, for the initiation of viral replication. The two truncated forms of the E2 protein, E2TR and E8/E2, function as repressors. They both lack an intact amino-terminal activation domain. All forms of the E2 protein are dimeric and their function requires preferential binding to a highly conserved palindromic target sequence⁶, termed E2-BS (Fig. 1b), which occurs 17 times in the BPV-1 genome.

Specific DNA binding is mediated through a carboxy-terminal 85-amino-acid domain which is also sufficient for dimerization^{7–9}. This domain is highly conserved among the E2 proteins of the papillomaviruses (30% identity) and does not include a sequence motif previously associated with DNA binding. The DNA-binding/dimerization domain is connected to a 160-amino-acid, acidic N-terminal activation domain through a proline-rich linker of variable length and composition termed the 'hinge' (Fig. 1a). This arrangement of domains is common to the E2 protein of all papillomaviruses.

We describe here the high-resolution crystal structure of the DNA-binding/dimerization domain, E2(326–410) of BPV-1, bound to a 16-base-pair (bp) DNA sequence which includes an idealized E2-binding site (Fig. 2). A dimer of E2(326–410) forms an eight-stranded antiparallel β -barrel made up of four strands from each subunit. A pair of α -helices symmetrically disposed on the outer circumference of the barrel contain all of the amino acids that are directly involved in base-sequence recognition. The DNA is bent smoothly around the barrel to enable successive major grooves to engulf these recognition helices.

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FIG. 1 Sequences of molecules used in the crystals. *a*, Sequence diagram showing the location of functional domains and sites of sequence identity/similarity between the DNA-binding domains of the E2 proteins from 10 papillomaviruses (adapted with permission from Giri and Yaniv⁸). Secondary structural elements seen in the crystal structure are indicated above the sequences. *b*, Oligonucleotide used in crystallization. The absolutely conserved sequence recognized by all papillomaviruses is underlined. Preferred nucleotides are in bold.



Crystallization and structure determination

An 85-amino-acid C-terminal fragment of the E2 protein, E2(326–410), previously reported to be necessary and sufficient for specific DNA binding, was cloned and overexpressed in *Escherichia coli*¹⁰. E2(326–410) was co-crystallized with the self-complementary oligonucleotide shown in Fig. 1*b*.

Rhombohedral crystals of the complex were obtained in the presence of lanthanide salts (SmCl₃, EuCl₃, GdCl₃, YbCl₃) and diffracted to at least 1.7 Å (Table 1). The crystallographic dyad coincides with the molecular dyad of the complex so that the asymmetric unit is made up of a single subunit of E2(326–410) and half a DNA duplex. Phases were obtained to 2.3 Å from the combination of a single isomorphous replacement and intensity differences arising in the parent data from the anomalous scattering of the lanthanide ion, which was bound to the protein. The heavy-atom derivative contained a 5-iodouracil in place of the thymine at position +2 (Table 1). These experimental phases produced a readily interpreted map. The structure was refined to a resolution of 1.7 Å with 98 water molecules and good stereochemistry (see Table 1 and Fig. 3 for details).

Structure of the protein

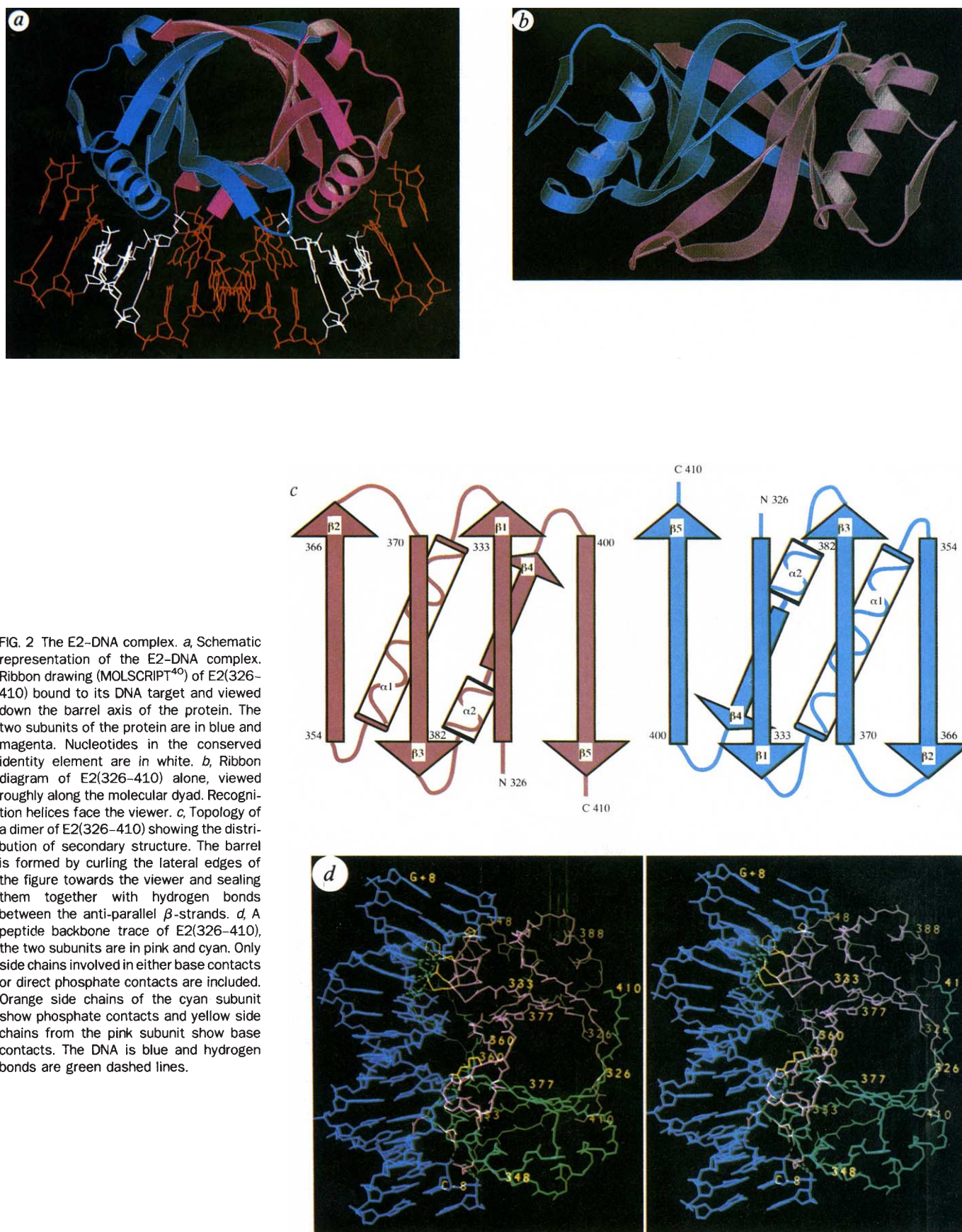
A unique folding pattern. The protein is a dyad-symmetric eight-stranded antiparallel β-barrel made up of two identical 'half-barrel' subunits (Fig. 2*b*). The distribution of secondary structure in E2(326–410) is schematically illustrated in Fig. 1 and Fig. 2*c*. Apart from the four β-strands per subunit that participate in barrel formation, each subunit has two α-helices that make crossover connections on the outside of the barrel. The larger and better conserved α-helix connecting β-strands 1 and 2 serves as the 'recognition helix'. Four of the five turns connecting secondary structural elements in each subunit of E2(326–410) are short and tightly constrained. The one much larger turn, connecting β-strands 2 and 3, reaches across the dyad to make a functionally important contact to the DNA that will be described later. The N termini, which attach the DNA-binding domain to the rest of the protein, are at opposite ends of the barrel, thus the hinge segments of the two subunits point in opposite directions away from the DNA and the dyad of the complex.

Dimer-stabilizing interactions. The E2 protein forms a very strong dimer that can be disrupted only under denaturing conditions such as 5 M urea¹¹. Dimerization leads to an extensive (2,567 Å²) solvent-excluded subunit interface¹². Three types of interactions are responsible for holding the subunits together. (1) A characteristic inter-backbone hydrogen-bonding network between the β-strands seals together the edges of each half barrel with a total of 10 hydrogen bonds. (2) Side chains, mostly from β-strands 2 and 5 which border each subunit, make inter-subunit hydrogen bonds. (3) There is an extensive hydrophobic core at the centre of the barrel containing a completely conserved Trp at position 360, which when replaced by a polar residue in mutational analyses disrupts the dimer¹⁰. Although not perfectly stacked, the indole rings of Trp 360 from opposing subunits are in van der Waals contact, thus accounting for their ability to be photocrosslinked¹⁰.

DNA conformation

In the E2/E2-BS complex, B-form DNA is wrapped smoothly around the protein barrel encompassing a pair of symmetrically disposed recognition helices in successive major grooves (Fig. 2). The DNA helix approximates a circle with a 45 Å radius. The straightest sections include base pairs 4, 5 and 6 which are the identity elements and the region of greatest contact with the protein's recognition helix. The bending pattern is in contrast to that seen in some specific complexes (CAP/CAP-site¹³, trp repressor/operator¹⁴) where the DNA bends mainly, and often quite sharply, in the region where the DNA's identity elements contact those of the protein. In the E2-BS, the largest deviation in the orientation of the base pairs from those of canonical B-DNA is the 14.0° roll between base pairs 3 and 4 (ref. 15). This is much less than the minimum 20° roll that unstacks the base pairs and thus defines a 'kink'¹⁶.

As one would expect from the continuous curvature, both the major and minor grooves are compressed on the concave side of the DNA that faces the protein. The width of the major groove that accommodates the recognition helix is compressed by 4.8 Å to 12.5 Å (shortest inter-phosphate distance) principally by positive roll angles. The minor groove that faces the protein in the centre of the target is compressed slightly by negative roll angles and a mean 4.3° overtwist of the central



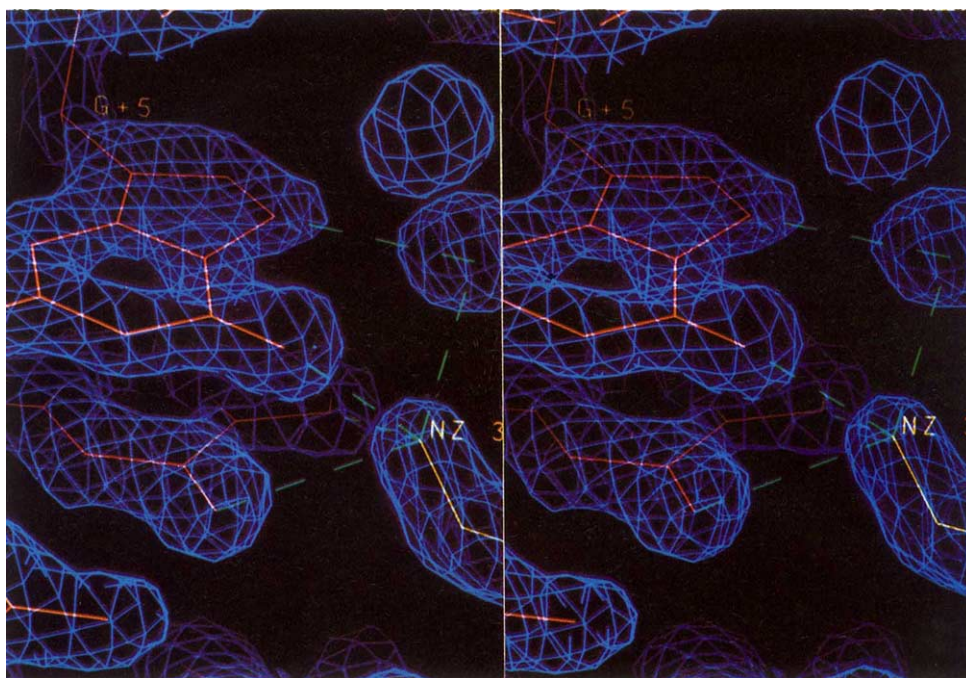
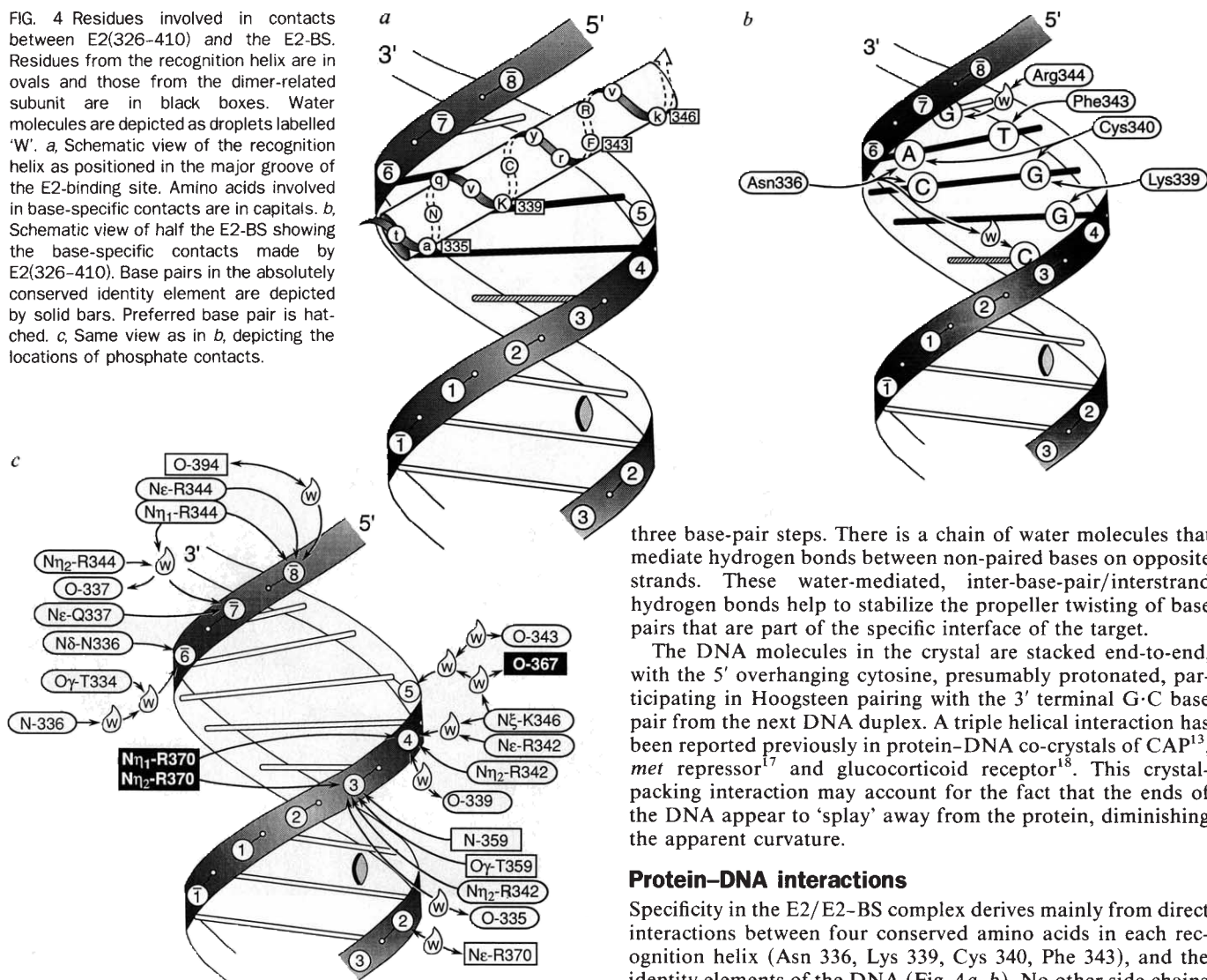


FIG. 3 Stereo view of the refined electron-density map ($3F_o - 2F_c$) showing the interactions of Lys 339 with G(+4) and G(+5). The map was calculated at 1.7 Å resolution and is contoured at 1.6 σ .

FIG. 4 Residues involved in contacts between E2(326–410) and the E2-BS. Residues from the recognition helix are in ovals and those from the dimer-related subunit are in black boxes. Water molecules are depicted as droplets labelled 'W'. *a*, Schematic view of the recognition helix as positioned in the major groove of the E2-binding site. Amino acids involved in base-specific contacts are in capitals. *b*, Schematic view of half the E2-BS showing the base-specific contacts made by E2(326–410). Base pairs in the absolutely conserved identity element are depicted by solid bars. Preferred base pair is hatched. *c*, Same view as in *b*, depicting the locations of phosphate contacts.



Protein-DNA interactions

Specificity in the E2/E2-BS complex derives mainly from direct interactions between four conserved amino acids in each recognition helix (Asn 336, Lys 339, Cys 340, Phe 343), and the identity elements of the DNA (Fig. 4*a, b*). No other side chains

contact the bases either directly or indirectly. Three of these amino acids make a total of seven direct hydrogen bonds to base pairs 4, 5 and 6 in the major groove. The fourth amino acid makes a hydrophobic van der Waals contact: Phe 343 with the 5'-methyl of T(+6). In addition, there are two specific water-mediated interactions to bases, ten direct phosphate contacts and 14 water-mediated phosphate interactions per DNA half-site. The base-discriminating interactions are indicated in Fig. 4*a, b* and depicted in detail in Fig. 5. Figure 4*c* schematically illustrates the phosphate interactions. It is evident that these contacts could not all occur without significant deformation of the DNA target, hence sequence-dependent deformation of DNA could play a role.

Three distinctive features of the E2/E2-BS interface. (1) Three of the four amino acids involved in base discriminating interactions straddle two base pairs. Asn 336 is shared by base pairs at positions 5 and 6, Lys 339 by positions 4 and 5, and Cys 340 by positions 5 and 6. Conversely, two of the three bases constituting a minimum E2 recognition half-site are contacted by more than one amino-acid side chain. Thus the side-chain orientation required for a specific hydrogen bond at one base is supported by another specific interaction. Specificity is further enhanced by the fact that the Asn 336 and Lys 339 side chains are also

buttressed by protein or phosphate interactions as is commonly seen in crystal structures of specific protein/nucleic acid complexes. (2) Cys 340 participates in a pair of discriminating interactions. The sulphhydryl group of Cys 340 donates a hydrogen bond to the O6 of G(+5), and is thus positioned to accept a hydrogen bond from the exocyclic 6-amino group of A(-6). Cysteine has not been reported to make specific base interactions in protein-DNA complexes. Cysteines are rarely observed in NH...S hydrogen bonds, except in the case of some metal coordinating proteins like the DNA-binding domain of the glucocorticoid receptor¹⁸, ferredoxin, rubredoxin and high-potential iron protein¹⁹. (3) The role of Asn 336 in base recognition is unlike that observed in the interaction of amide side chains with DNA in the *engrailed* homeodomain²⁰, λ repressor²¹ and 434 repressor complexes²². In all these cases, the amide group was found hydrogen-bonded to both the N7 and the exocyclic 4-amino groups of the same adenine, as suggested by Seeman *et al.*²³. In the E2/E2-BS complex, the Asn 336 side chain contacts successive bases A(-6) and C(-5), hydrogen-bonding with the N7 of A(-6) and the exocyclic 4-amino group of C(-5). Asn 336 also hydrogen-bonds with C(+3) through a water molecule, thereby spanning four base pairs.

Although the central four base pairs of the E2-BS are not

TABLE 1 Experimental data on crystal structure analysis and refinement

(a) Quality of MIR phases								
Resolution limit (Å)	10.2	6.8	5.2	4.1	3.4	3.0	2.6	2.3
Joint phasing power*	2.44	3.46	3.71	2.83	2.45	1.98	1.84	1.34
(b) Refinement summary								
Resolution limit (Å)	5.0	4.0	3.2	2.8	2.4	2.0	1.7	Total
Predicted†	524	1,116	2,220	2,232	4,120	7,664	11,292	29,018
Refined (all data)	468	1,069	2,042	2,083	3,649	6,138	7,349	22,798
R-factor‡ (%)	18.0	12.6	13.4	19.0	20.6	21.5	29.4	19.4
Refined (>1.5 σ)	464	1,053	2,024	2,002	3,443	5,583	5,096	20,475
R-factor‡ (%)	20.1	13.4	13.7	17.4	17.4	16.5	18.9	16.3

E2 (326–410) was overexpressed in the *Escherichia coli* strain BL21(DE3)pLysS. The cells were induced when they reached an absorbance at 600 nm (A_{600}) of 0.4 with 0.4 mM isopropyl- β -D-thiogalactoside (IPTG). One hour after induction the cells were collected, frozen in a dry-ice/ethanol mixture and stored at -80°C . Before purification, the cells were thawed and sonicated in 25 mM Tris-HCl pH 7.5, 100 mM NaCl, 10 mM dithiothreitol (DTT), 0.5 mM EDTA and 1 mM 4-methylphenylsulphonyl fluoride (PMSF). The cell lysate was purified on a Fast-flow S-Sepharose (Pharmacia) column with a gradient of 400 to 700 mM NaCl in 20 mM 2-(N-morpholino)ethanesulphonic acid (MES), pH 6.2. The fractions containing E2(326–410) were made 10 mM in DTT, concentrated and loaded on a P-60 (Bio-Rad) gel filtration column. E2(326–410) was eluted with 25 mM Tris-HCl, pH 7.5, 100 mM NaCl as a dimer, and produced a single band on electrophoresis of overloaded, silver-stained denaturing gels (incorporating sodium dodecylsulphate). The protein fractions were stored at -80°C in 10 mM DTT at concentrations below 2.2 mg ml^{-1} or as a complex with DNA. Oligonucleotides were synthesized by the phosphoramidite method and obtained with 5' dimethoxytrityl blocking groups attached (J. Flory, Howard Hughes Medical Institute, Yale). They were purified by reversed-phase HPLC on a C4 resin (Vydac), detritylated in acetic acid and then purified by FPLC at pH 11.8 with a Mono-Q matrix (Pharmacia). DNA concentrations were estimated using extinction coefficients of the individual nucleosides corrected for the hypochromic effect. Before crystallization, the uncomplexed protein samples were thawed and mixed with near-stoichiometric amounts of the oligonucleotide (molar ratio of protein dimer: oligonucleotide duplex, 1:1.2). The complex was concentrated in a Centricon (Amicon) to $16\text{--}20\text{ mg ml}^{-1}$ complex. Storage of E2(326–410) alone at high concentration resulted in the formation of polymeric species (on denaturing gel electrophoresis) that were not observed on storage of the protein-DNA complex at high concentrations. Crystals were grown by vapour diffusion in hanging drops. Drops containing 11.7 mg ml^{-1} protein-DNA complex, 28.8 mM Tris-HCl, pH 7.5, 75 mM NaCl, 9.25% polyethyleneglycol (PEG) 3350, 12.5 mM KCl, 0.5 mM CaCl_2 and 1.5 mM lanthanide chloride were placed over reservoirs of 35–37% PEG 3350, 40 mM Tris, pH 7.5, 50 mM KCl, 2 mM CaCl_2 . Crystals appeared in 2–5 days at room temperature and typically grew to $0.4 \times 0.4 \times 0.8\text{ mm}$ in 2 weeks. The crystals belonged to the space group $R32$ with $a=b=c=64.6\text{ Å}$, $\alpha=\beta=\gamma=61.8^\circ$ and diffracted to 1.7 Å . The asymmetric unit of the E2(326–410)/E2-BS crystal consists of half a DNA duplex and one subunit of the protein. All data were collected at room temperature on a San Diego Multiwire System area detector. Intensities were scaled with SCALEPACK (Z. Otwinowski). All of the crystals used in the structure solution included lanthanide ions, either Eu^{3+} or Yb^{3+} . The structure was initially solved with 2.3 Å data from europium-containing crystals. Patterson maps were calculated from the Bijvoet differences arising in the parent data due to the anomalous scattering of the Eu^{3+} ion, and revealed one major (97% occupancy) and two minor sites (9% and 6% occupancy). The positions and occupancies of these atoms were refined³⁴ and a bimodal phase probability distribution was calculated. The isomorphous heavy-atom derivative contained the fully occupied Eu site of the parent and one fully occupied iodine site (replacing the 5-methyl group of T(+2)). In addition, the minor Eu sites were more heavily substituted than the parent. A second phase probability was calculated using the isomorphous and Bijvoet differences of the derivative. The figure of merit weighted centroid phases of the joint probability distributions were used to calculate an initial electron-density map at 2.3 Å , which was clearly interpretable and a model was built using FRODO³⁵. Initial refinement was done by simulated annealing³⁶. Final refinement was done on the 1.7 Å Yb-containing parent data with conventional least-squares methods using NUCSQ and PROFFT^{37–39}. The agreement between the calculated and observed X-ray structure factors of the final model including 98 water molecules is 19.4% for all data between 6 and 1.7 Å , and 16.3% for data stronger than 1.5σ between 6 and 1.7 Å . The average deviation of the bond distances and interbond angles from standard values is 0.02 Å and 2.7° , respectively. ϕ , ψ backbone rotations of all non-glycine residues fall within conventional limits.

* $\sum_n |F_c^o(h)|^2 / \sum_n |F_p^o(h)|^2 \exp(i\phi_c) + F_H^o - |F_H^o|$, where F_H^o is the calculated structure factor of the heavy-atom constellation, $|F_p^o|$ and $|F_H^o|$ are the observed amplitudes for the protein and heavy-atom derivative, respectively, ϕ_c is the calculated phase of the parent structure, and the sum is over all observations.

† Friedel mates were kept separate.

‡ R-factor $\sum_n ||F^o| - |F^c|| / \sum_n |F^o|$, where $|F^o|$ is the observed structure amplitude and $|F^c|$ is the structure amplitude calculated from the refined coordinates.

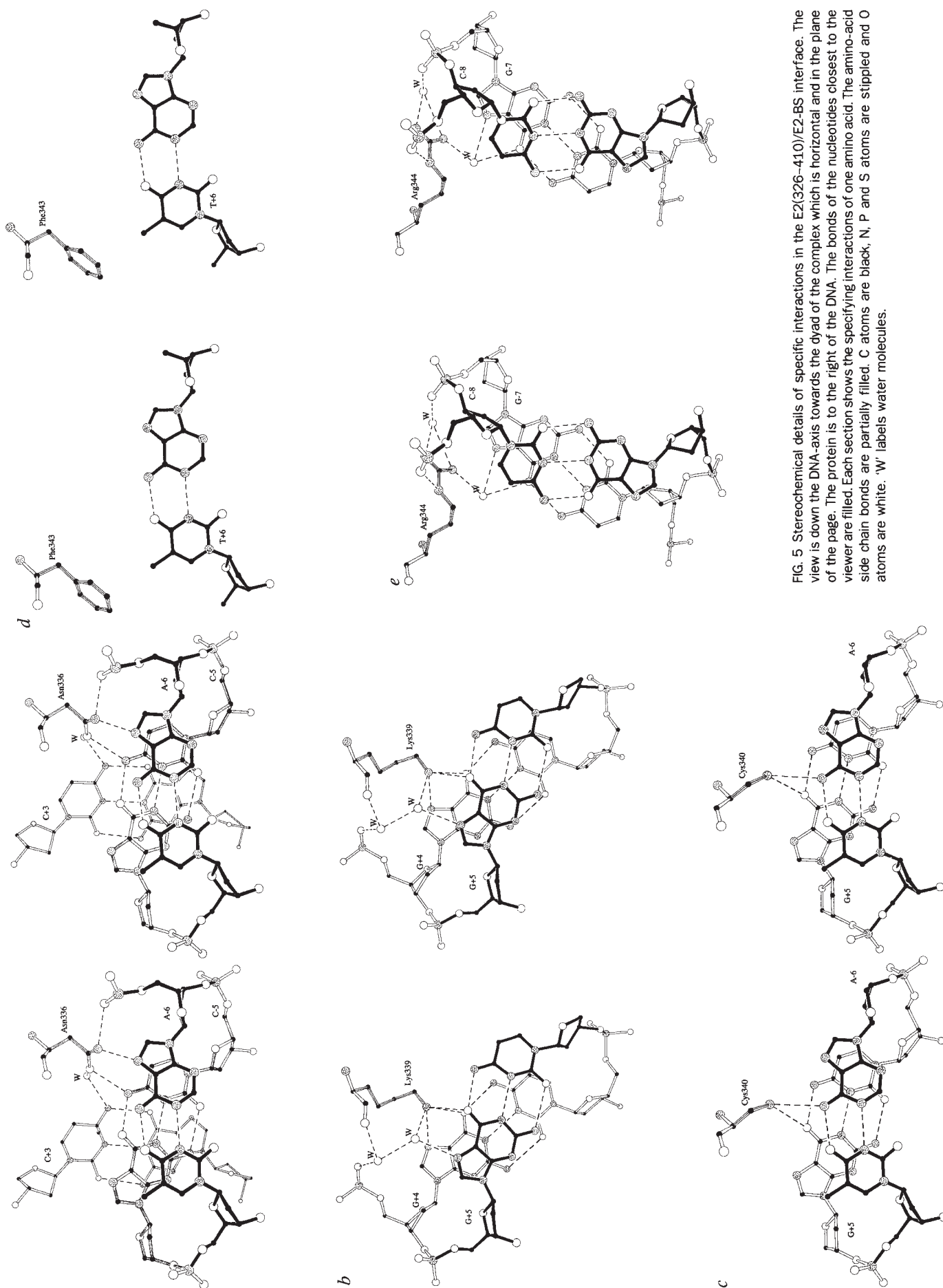


FIG. 5 Stereochemical details of specific interactions in the E2(326-410)/E2-BS interface. The view is down the DNA-axis towards the dyad of the complex which is horizontal and in the plane of the page. The protein is to the right of the DNA. The bonds of the nucleotides closest to the viewer are filled. Each section shows the specifying interactions of one amino acid. The amino-acid side chain bonds are partially filled. C atoms are black, N, P and S atoms are stippled and O atoms are white. 'W' labels water molecules.

contacted by the protein in the minor groove, there are extensive contacts between the protein and the phosphates in this region. The sugar-phosphate backbones of the central six nucleotides run antiparallel to β -strand 2 of both protein subunits. They are held to the protein by 12 protein-phosphate hydrogen bonds, six in each 'half complex'. Arg 370 in the elongated loop between β -strands 2 and 3 reaches across to the opposing half of the DNA target. The terminal guanidino group makes hydrogen bonds to the bridging O3' of the sugar at position +3 and the minor groove non-bridging phosphate oxygen at position +4. Protein-phosphate interactions through bridging oxygens are rare, having been reported only for Arg 43 in the 434 repressor-DNA complex²². Residue 370 in all the papillomaviruses is either an Arg or Lys, underscoring the functional importance of this interaction. Additionally, the γ -OH and backbone NH of Thr 359 contact phosphate +3 in the region of the only two β -bulges of the E2 β -barrel that cause the twist of β -strand seen in Fig. 2b.

A water molecule mediates the hydrogen-bonded contact between the exocyclic 4-amino group of C(+3) and the side-chain oxygen of Asn 336 that could contribute to increased affinity. Base pair (G·C(-3)) is not absolutely conserved, but it occurs in 12 out of the 17 E2-BS in the BPV-1 genome²⁴ and is associated with enhanced DNA-binding affinity. Base pair (G·C(-7)), however, is not at all part of the identity sequence, yet Arg 344 is hydrogen-bonded through a single water molecule to both the N7 and O6 of its G. The side chain of Arg 344 is also buttressed by other hydrogen bonds to phosphates -8 and -7.

Discussion

The crystal structure of the E2/E2-BS complex has three distinguishing features: (1) it reveals a new structural scaffold for presenting recognition helices to successive major grooves of a specific DNA target; (2) the dimerization interface of the protein is a previously undescribed association of domed β -sheets into a barrel; and (3) DNA wraps around the protein barrel in a relatively smooth fashion so that the DNA-helix axis achieves an overall 45 Å radius of curvature principally by bending the central base pairs towards the minor groove.

A dimeric β -barrel has not been previously reported in protein structures. Although β -sheets are often involved in dimerization interfaces (for example insulin²⁵), this is the first occurrence of dimerization of domed β -sheets sealed together at both edges by hydrogen bonds typical of antiparallel β -structure. E2 is a preformed dimer in solution¹⁰ like *trp* repressor¹⁴, *met* repressor¹⁷ and CAP¹³, but unlike *arc* repressor²⁶, glucocorticoid receptor¹⁸ and GAL4 (ref. 27), which dimerize on binding to DNA.

The E2 dimer orients the DNA-recognition helices so that the α -helical axes track the major grooves of the target. Although the original proposal for how the helix-turn-helix motif was likely to interact with DNA showed the α -helix tracking the major groove²⁸, structural studies of DNA-protein complexes showed a range of orientations usually with the recognition helix more perpendicular to the DNA helical axis than that seen in the E2/E2-BS complex. These differences in the orientation of the recognition helix may influence the way the bound DNA target will bend. For example, when bound by the CAP¹³ and *trp* repressor¹⁴, their symmetrical DNA targets are bent twice, once in the middle of each half-site, by 45° and 14°, respectively. The bends are towards the major groove where the specifying interactions take place and, in both cases, the bend is sharp, being accomplished principally in one inter-base-pair step. In contrast, Fig. 2d shows that in the E2/E2-BS complex, the DNA is bent least in the regions of the DNA where E2 makes specific contacts. Instead, there is an almost continuous bend towards the minor groove, smoothly distributed over the central four base pairs. The different bending mechanics exhibited by the E2/E2-BS system may reflect the way the recognition helices interact with the DNA. In the cases cited above where the DNA

bends in the region of specific contacts, the recognition helix is almost perpendicular to the DNA, giving the DNA a fulcrum around which to bend. In the E2/E2-BS complex, however, the recognition helix is aligned with its axis along the major groove, thus acting as a reinforcing element that maintains a straight DNA segment.

If the specific contact regions of the E2-BS are straight, then to complement the convex surface of the protein, the centre of the target DNA is forced to bend towards the minor groove. The severity of the bend is imposed by the closeness with which the recognition helices are held to the outside of the barrel. This bend is associated with a consistent trend in roll angle in which the edges of the bases facing the protein pinch together. The full influence of E2 on the bend of the E2-BS may not be represented here, because, as in the case of the CAP/CAP-site complex¹³, the ends of the target flare away from the protein in making end-to-end base-stacking interactions which stabilize the lattice.

Amino-acid conservation in the E2 DNA-binding domain is noticeably clustered in three regions (Fig. 1a); the recognition helix (332-346), the Thr-Thr-Thr-Trp sequence between 357 and 360, and the helix-containing connector between β -strands 3 and 5 (385-396). Amino-acid identity in the recognition helix applies to the five amino-acid residues that make specific contacts with the E2-BS. Clearly there is a need to conserve both the nature and location of functional groups in the substructure that forms the specific interface between protein and DNA. Of particular note is the absolute conservation of Asn 336 which plays a major role in DNA recognition by making specifying contacts to three bases, and one supporting contact with a phosphate. It is also located at the third position of an α -helix which is a favoured position for Asn because of its ability to form a stabilizing 'cap' on the N terminus of the helix^{29,30}. We suggest that the side chain of Asn 336 rearranges and 'caps' the recognition helix in the absence of DNA. Residue 333 must be Gly to accommodate the sharp change in direction at the end of β -strand 1 with backbone torsion angles that are unacceptable to other amino acids. The Thr-Thr-Thr-Trp sequence contacts DNA phosphates in the centre of the complex through a β -bulge (Thr 359) while contributing hydrogen bonds and van der Waals interactions (Trp 360) to the dimer interface. Replacement of the Thr with a Ser, as seen in some papillomaviruses, does not disrupt any of these interactions. Hydrophobic residues are conserved in the connection between strands β 3 and β 5 to bind this connector to a hydrophobic patch on the surface of the β -barrel. Additionally, the guanidino group of Arg 386 forms an ion-pair with the side chain of Asp 409 of the dyad subunit, further cementing the dimer interface.

The large number of base-discriminating interactions in the E2/E2-BS complex and the interdependence of their stereochemistry account for the absolute conservation of the target's identity elements and the side chains that contact them. The extensive 'interwoven' network of specific interaction coupled with the 48 phosphate contacts (20 direct and 28 water-mediated) per complex are consistent with the high DNA-binding affinity ($K_d = 4 \times 10^{-10}$ M)³¹. The complementary deformation of DNA seen in the crystal structure allows both recognition helices of the protein to interact symmetrically with the palindromic E2-BS and is in agreement with the electrophoretic observation of E2-induced DNA-bending³². This bend, in turn, may also contribute to the proposed role of full-length E2 in mediating long-range interactions through looping³³. □

Received 22 June; accepted 7 September 1992.

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ACKNOWLEDGEMENTS. We thank Z. Otwinowski (HIMI/Yale) and E. Androphy (Tufts Medical School) for their advice in some of the experiments. Work at Yale was supported in part by the NIH. R.H. was supported in part by the National Cancer Center. The coordinates of the E2(326–410)/E2-BS complex will be submitted to the Brookhaven Protein Data Bank.

LETTERS TO NATURE

A cluster of protogalaxies at redshift 3.4?

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USON *et al.*¹ have detected the 21-cm hydrogen line at a redshift of $z = 3.4$, both in absorption against a radio galaxy, 0902 + 343, which is also at $z = 3.4$, and more interestingly in emission from a nearby region at the same redshift. They interpret the H I emission as arising from a Zeldovich ‘pancake’², a primordial supergalactic condensation of gas which has yet to fragment into protogalactic units. Pancake (or ‘top-down’) theories of galaxy formation have several well-known difficulties^{3,4}, however, and ‘bottom-up’ theories, in which protogalactic units form first and only later aggregate into clusters, are currently more popular. We propose that the H I emission seen by Uson *et al.* may be emission from neutral hydrogen in a collection of protogalaxies, which are of the type needed to explain the damped Lyman- α absorption systems in quasar spectra⁵. For this explanation to work, more galaxies per unit mass must form in protocluster environments than in the field. The narrowness of the observed emission line implies that the protocluster is near gravitational turn-around, the moment at which it detaches itself from the general cosmic expansion and begins to collapse. The absorption line can arise from a gaseous halo rich in H I around the radio galaxy, or from other galaxies in the vicinity.

The H I mass of a cloud emitting 21-cm line radiation can be estimated⁶ from the peak flux density S_ν and frequency width $\Delta\nu_0$ (full width at half maximum for a gaussian profile) of the observed emission line using

$$M_{\text{H I}} \approx 10^{13} M_\odot h^{-2} \left(\frac{S_\nu}{5.3 \text{ mJy}} \right) \left(\frac{\Delta\nu_0}{1 \text{ MHz}} \right) \times \left[\frac{(\Omega_0 z + (\Omega_0 - 2)((1 + \Omega_0 z)^{1/2} - 1))^2}{\Omega_0^4} \right] \quad (1)$$

Here z is the cloud redshift, Ω_0 the density parameter of the Universe, $M_\odot$ the solar mass, and the Hubble constant is $H_0 = 100h \text{ km s}^{-1} \text{ Mpc}^{-1}$. We have assumed the cloud to be optically thin, which is generally valid. The H I emission line detected by Uson *et al.* has $S_\nu = 11.4 \pm 2.3 \text{ mJy}$, and $\Delta\nu_0 \approx 196 \text{ kHz}$ corresponding to a velocity width of $\Delta v \approx 180 \text{ km s}^{-1}$ at $z = 3.3970 \pm 0.0003$. For these values equation (1) implies $M_{\text{H I}} \approx 2.2 \times 10^{13} h^{-2} M_\odot$ if $\Omega_0 = 1$, and $M_{\text{H I}} \approx 6.9 \times 10^{13} h^{-2} M_\odot$ if $\Omega_0 =$

0.1. As the source is slightly resolved with an observed extent¹ $\Delta\phi \approx 300''$, larger than the angular resolution $\sigma_{\text{beam}} \approx 200''$, $M_{\text{H I}}$ could be larger, by a factor of $\sim (\Delta\phi/\sigma_{\text{beam}})^2 \approx (300/200)^2 \approx 2.25$. The total mass of the object M_T is likely to be even larger, by a factor of $F \approx 10$, when one takes into account the associated mass in ionized gas and dark matter. Such large values of $M_{\text{H I}}$ and M_T raise two potential problems.

First, it is well known that any smoothly distributed hydrogen in the Universe, at $z = 3-4$, is likely to be almost completely ionized⁷⁻⁹. Of course, neutral hydrogen can be regenerated from the intergalactic medium during gravitational collapse provided gas cooling is efficient. In the canonical hierarchical clustering theories, however, galaxies could have collapsed by $z = 3.4$, but not rich clusters¹⁰. So what is the nature of the emitting object with a mass M_T as large as that of a rich cluster?

Although the smooth intergalactic medium at high z lacks H I, quasar spectra do show many absorption lines indicating the existence of neutral hydrogen in clumps. Particularly important in the present context are the ‘damped Ly α systems’^{5,11-13}. These absorbers may contribute, at their observed redshift of $\sim 2-4$, as much mass in neutral hydrogen as the total stellar content of all present-day disk galaxies^{5,11-13}. As discussed below, the object seen by Uson *et al.*¹ may then be explained as a large protocluster (or collection) of such absorbers.

Let us estimate the H I contributed by the damped Ly α systems to any protocluster-sized volume¹³. Suppose the absorbers are uniformly distributed with a comoving space density n_0 and typical radius r_c . Then their expected number per unit redshift is $(dN(z)/dz) = \pi r_c^2 \times n_0 (1+z)^3 \times (c dt/dz)$. Also the H I mass of a typical absorber $m_{\text{H I}} = (4\pi/3) r_c^3 n_{\text{H I}} m_p \approx (\frac{4}{3}) \pi r_c^2 N_{\text{H I}} m_p$, where $N_{\text{H I}} = n_{\text{H I}} r_c$ is the observationally measurable average H I column density of the absorber. Consider a volume V of the background Universe, which contains a total mass $M_T = \rho_c (1+z)^3 \Omega_0 V$, where $\rho_c = (3H_0^2/8\pi G)$ is the critical density. The mass of H I contributed by the absorbers to this volume is simply $M_{\text{H I}} = n_0 (1+z)^3 m_{\text{H I}} V = (n_0 m_{\text{H I}} / \Omega_0 \rho_c) M_T$. So, in terms of observationally determined quantities

$$M_{\text{H I}} = E \times \frac{4}{3} \frac{m_p N_{\text{H I}}}{\Omega_0 \rho_c} \left(\frac{H_0}{c} \right) \frac{dN}{dz} \frac{(1 + \Omega_0 z)^{1/2}}{(1+z)} \times M_T$$

$$\approx 3.1 \times E \times 10^{12} h^{-2} M_\odot \left(\frac{dN/dz}{0.5} \right) \left(\frac{N_{\text{H I}}}{10^{21} \text{ cm}^{-2}} \right)$$

$$\times \left(\frac{M_T}{10^{15} h^{-1} M_\odot} \right) \quad \text{for } z = 3.4; \Omega_0 = 1 \quad (2)$$

Here we have taken into account the possibility that the number density of absorbers may be enhanced by a factor of E in regions

such as protoclusters compared with the general background. (We have also adopted the value of M_T estimated for the Coma cluster¹⁴.) The damped Ly α systems have an average $N_{\text{H I}} \approx 10^{21} \text{ cm}^{-2}$. Earlier estimates¹¹ gave $dN/dz = 0.29 \pm 0.08$ at an average redshift $\langle z \rangle = 2.4$, but later work¹³ indicates $0.16 \leq dN/dz \leq 0.25$ at $\langle z \rangle \approx 2.5$. The only data directly applicable to the redshift of the Uson *et al.* object indicate¹³ $dN/dz = 10^{-0.25} \approx 0.56$, although with large error bars (see Fig. 7 of ref. 13). For $dN/dz \approx 0.3$ – 0.6 and $M_T \approx (1-2) \times 10^{15} h^{-1} M_\odot$, we see from equation (2) that we require $E \approx 5$ – 20 if we are to explain this object as a protocluster of absorbers, of the kind that produce the damped Ly α lines in quasars. If the absorbers are protogalaxies, which seems likely^{11-13,15,16}, this in turn requires that an enhanced number of galaxies per unit mass form in a protocluster compared with the field, even before the protocluster collapses.

The idea that galaxy formation could be more efficient in protoclusters has support from both theory and observations. For example, in biased galaxy formation theories, galaxies are born clustered^{17,18}. Bardeen *et al.* emphasize¹⁸ that a protocluster at high z could have a strong enhancement of galaxy density even though the underlying mass density contrast was small at that time. For a cold dark matter model with $\Omega_0 = 1$, they numerically estimate an enhancement by $E \approx 3$ – 10 for a typical Abell cluster forming now; the value depends on how they smooth the density field. In addition galaxies may form more efficiently in protoclusters purely because of hydrodynamic and gas cooling effects¹⁹. The observed properties of rich clusters can also be used to estimate²⁰ E (ref. 20). If the number of galaxies per unit mass forming in a protocluster is indeed enhanced by a factor of E compared with the field, then when the cluster collapses (and if the galaxies do not segregate from the mass), the excess density contrast in galaxies ($\delta N/N$) will also be a factor of E larger than the mass contrast, ($\delta M/M$). So

$$E = \frac{\delta N/N}{\delta M/M} = \left(\frac{N_g}{n_b V_a} \right) \left(\frac{M_{\text{cl}}}{\rho_b V_a} \right)^{-1} = \frac{N_g \langle L \rangle}{M_{\text{cl}}} \frac{\rho_c}{n_b \langle L \rangle} \Omega_0$$

$$= \frac{(M/L)_{\text{critical}}}{(M/L)_{\text{cluster}}} \Omega_0 \quad (3)$$

Here N_g is the number of galaxies in a cluster of volume V_a and mass M_{cl} ; n_b the average galaxy number density in the field and ρ_b the background density of the Universe. We have multiplied and divided by the average luminosity of a cluster galaxy $\langle L \rangle$. The ratio of cluster mass to light $(M/L)_{\text{cluster}}$ ranges between ~ 200 and $400 h (M_\odot/L_\odot)$, and the critical ratio for a universe with closure density $(M/L)_{\text{critical}} \approx 1,500 h (M_\odot/L_\odot)$ (compare with ref. 21). Using equation (3) gives an estimate of $E \approx 3.75$ – 7.5 , for a $\Omega_0 = 1$ universe (we assume $\Omega_0 = 1$ henceforth). Therefore it seems that the enhancement of $E \approx 10$ required to explain the detection by Uson *et al.* is near the upper limit of that expected, but is plausible.

We now show that the gas-rich protogalaxies of our model can remain largely neutral in spite of ionization during star formation, because such ionization is offset by recombination. The luminosity of ionizing photons, L_1 , from hot stars in a protogalaxy can be estimated by relating it to the rate of metal production in the galaxy^{22,23}. Songaila *et al.*²² estimate an energy output in ionizing photons per unit mass of returned metals, $\epsilon_\nu \approx 1,100 \phi (\nu/\nu_L)^{-1} \text{ erg g}^{-1} \text{ Hz}^{-1}$, between the Lyman limit ν_L and the He I edge at $504 \text{ \AA}$, where $\phi \approx 1$ for a Salpeter-type initial mass function of the stars. Suppose R is the rate of star formation in solar masses per year, and $p \approx 1.4\%$ is the mean yield of metals, then we get $L_1 = \int p(R \times 1 M_\odot \text{ yr}^{-1}) \epsilon_\nu d\nu \approx 2 \times 10^{42} R \text{ erg s}^{-1}$. Next we compare L_1 with the rate of energy loss L_L due to recombinations. Consider a protogalaxy with a hydrogen mass m_H lying in a disk, as envisaged by Wolfe *et al.* for damped systems, of radius r and height z . The average electron and proton densities for completely ionized gas

are $n_e = n_p \approx (m_H/m_p \pi r^2 z)$. Therefore $L_L \approx h \nu_L \alpha_H n_e n_p \pi r^2 z \approx 2 \times 10^{45} \text{ erg s}^{-1} (m_H/10^{11} M_\odot)^2 (r/30 \text{ kpc})^{-2} (z/1 \text{ kpc})^{-1} (T/10^4 \text{ K})^{-3/4}$. Here α_H is the recombination coefficient, m_p is the proton mass and h is Planck's constant. (A similar value for L_L is also obtained for a spherical protogalaxy of radius $r \approx 10 \text{ kpc}$). Comparing L_L with L_1 , we see that for gas-rich protogalaxies, ionization by stars cannot overcome recombinations, unless $R \geq 300$. Such large values of R are unlikely if the protogalaxies resemble the damped Ly α systems, as envisaged in our model. First, the damped systems are inferred to contain largely unprocessed gas with a metal abundance $Z \leq 0.1 \text{ solar}^{11,24}$. If a protogalaxy has been making stars at the rate R for a time $t \approx 10^9 \text{ yr}$, comparable to the Hubble time at these redshifts, then the resulting $Z \approx (pRt/m_H)$ will exceed 0.1 solar unless $R \leq 15$. Second, the lack of Ly α emission from damped systems such as PHL957 implies²⁵ $R \leq 5 h^{-2}$. Finally, even in the damped system where Ly α emission has been detected, one infers¹⁶ $\dot{Z} \leq 5.45 \times 10^{-13} \text{ yr}^{-1}$, corresponding to $R \approx (Z m_H/p) \leq 4$. Hot stars are therefore unlikely to ionize the hydrogen substantially. Also, any metagalactic ionizing photons with a flux of, say, $J = J_{21} 10^{-21} \text{ erg s}^{-1} \text{ cm}^{-2} \text{ Hz}^{-1} \text{ sr}^{-1}$ (where $J_{21} \approx 1$; compare with ref. 26) penetrate a distance of only $l \approx 0.4 \text{ pc} (n/1 \text{ cm}^{-3})^{-2} J_{21}$. The protogalaxies in our model can therefore be taken to be substantially neutral (see also ref. 22).

We will consider the constraints imposed by the observed velocity width Δv and angular extent $\Delta \phi$ of the emission region. From Uson *et al.*¹, we have $\Delta v \approx 180 \text{ km s}^{-1}$ and $\Delta \phi \approx 5 \text{ arcmin}$. Δv is much smaller than the Hubble expansion velocity $v_H \approx 960(1+z)^{1/2} (M_{15})^{1/3} \text{ km s}^{-1}$ of a spherical shell containing a cluster-sized mass (we have defined $M_{15} = (M_{\text{cl}}/10^{15} h^{-1} M_\odot)$). The protocluster is therefore sufficiently overdense to slow its expansion compared with the Hubble flow. The spherical top hat model²⁷ can be used to estimate the velocity v , and the proper size r at $z = 3.4$, of a shell containing a mass M_{cl} and collapsing at a redshift z_{coll} . For $z_{\text{coll}} = 0$, we get $v \approx 1,440 (M_{15})^{1/2} \text{ km s}^{-1}$ and $r \approx 1.9 h^{-1} (M_{15})^{1/3} \text{ Mpc}$ corresponding to $\Delta \phi \approx 9.1 \text{ arcmin}$. For a higher collapse redshift of 1.5 , the corresponding numbers are $v \approx 260 (M_{15})^{1/3} \text{ km s}^{-1}$, $r \approx 1 h^{-1} (M_{15})^{1/3} \text{ Mpc}$ and $\Delta \phi \approx 4.8 \text{ arcmin}$. In the latter case the protocluster is near turn-around at $z \approx 3$. The linewidth depends on the density profile of the protocluster. For a rough estimate, however, we may take $\Delta v \approx v$. It seems that one needs a relatively large $z_{\text{coll}} (\approx 1.5)$, to satisfy the velocity constraint. This redshift is larger than the value of $z \approx 1$ obtained for the most distant clusters discovered so far²⁸. We may, however, be observing the turn-around of the short axis of the protocluster at $z_s \approx 3$, whereas the other axes collapse only at low redshifts $z_1 \approx 0$.

Consider now the 21-cm absorption detected in front of the radio galaxy 0902+343. This line occurs at a redshift $z_{\text{abs}} = 3.3968 \pm 0.0004$, with a peak optical depth $\tau = 0.0089 \pm 0.0014$, and $\Delta v = 270 \pm 50 \text{ km s}^{-1}$. We then derive $N_{\text{H I}} \approx 4.7 \times 10^{21} \text{ cm}^{-2} (T_s/10^3 \text{ K})$ (see also ref. 1), implying that the H I mass of the absorber $M_{\text{abs}} \approx 1.1 \times 10^{10} M_\odot (r/10 \text{ kpc})^2 (T_s/10^3 \text{ K})$, assuming that it has a transverse radius r . (Here T_s is the spin temperature of the gas.) Note that Δv is much larger than the thermal velocity for H I which is $\leq 10 \text{ km s}^{-1}$. It must therefore arise from bulk motions caused by gravity or turbulence. This virtually rules out a small mass cloud as the absorber; more plausible is to associate the absorbing gas with a halo around the radio galaxy itself or a collection of intervening galaxies. Such gaseous halos may be crucial to understanding the optical emission around high- z radio galaxies²⁹ and in confining these radio sources³⁰.

The detection of a protocluster in emission near turn-around with $\Delta v \approx 200 \text{ km s}^{-1}$ is likely to be rare because a cluster spends much less time in this phase than during initial expansion or collapse after turn-around with a larger $\Delta v \approx 1,000 \text{ km s}^{-1}$. In the latter, more probable case, the peak flux will go down by ~ 5 for the same $M_{\text{H I}}$, but if a matched filter is used, the signal-to-noise ratio will decrease by only $\sqrt{5}$ (see ref. 31 where

the easier detection of structures near turn-around has been emphasized). To detect more such H I protoclusters, we require searches over a larger volume of space and/or considerable improvement in observational sensitivity³². A testable prediction of our model is that one should see substructure in the protocluster, at the level of individual or groups of galaxies, a few tens of arcseconds (a few hundred kiloparsecs) in angular resolution. This is much smaller than the resolution of the Very Large Array as used by Uson *et al.*¹. Observations with higher resolution will require much larger integration. Equally it would be very difficult to make out substructure in velocity space, because the emission linewidth of an individual protogalaxy is expected to be $\leq 100 \text{ km s}^{-1}$, not much smaller than the linewidth seen in the Uson *et al.*¹ object. It may be more rewarding to search for Ly α emission of the kind recently seen from a damped Ly α absorber¹⁶ (see above). Clustering of the damped systems would also support our model. Companion galaxies to the damped Ly α systems have been detected in two cases^{25,33}, one in fact having $z = 3.4$. Finally we note that the angular separation of ~ 33 arcmin between the emitting and the absorbing regions corresponds to a co-moving separation $\sim 30.3 h^{-1} \text{ Mpc}$, comparable to the size of large-scale structures seen in the Center for Astrophysics slices of the Universe³⁴. $\square$

Received 2 January; accepted 3 August 1992.

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ACKNOWLEDGEMENTS. We thank R. Sinha for comments on the manuscript and V. Kapahi for discussions.

Gamma-ray bursts from high-velocity neutron stars

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RECENT observations with the BATSE instrument on the Compton Gamma Ray Observatory show that the distribution of γ -ray bursts is isotropic but radially non-uniform¹. Spectral features, time histories and the presence of X-ray tails suggest that the bursts arise from galactic neutron stars², but low-velocity neutron stars born in the galactic disk would be concentrated towards the galactic plane^{3,4}, which would not fit the BATSE results. Neutron stars born with velocities greater than $\sim 800 \text{ km s}^{-1}$ will, however, escape from the Galaxy's gravitational field. We show here that a population of such objects can fit the γ -ray burst distributions found by BATSE and also the Pioneer Venus Orbiter⁵, although two important conditions must be met: the high-velocity neutron stars should turn on as burst sources only after some time (perhaps after they have ceased to be radiopulsars), and the low-velocity neutron stars must rarely generate γ -ray bursts. The observed correlation^{6–8} in pulsars between magnetic moment and velocity may provide a physical cause for the difference in bursting properties between the two populations. Our model implies that the brightest bursts, with fluxes $\geq 3 \times 10^{-5} \text{ erg cm}^{-2} \text{ s}^{-1}$, will be anisotropically distributed.

Because no γ -ray burst (GRB) event has been convincingly identified with a stellar or extragalactic counterpart⁹, bursts are probably associated with isolated neutron stars from the Galaxy. Core collapse of type II supernovae is generally thought to produce isolated neutron stars which can be observed as

radiopulsars for $\sim 10^7$ years after their birth. Very-high-velocity neutron stars could be formed in the supernova event, and many radiopulsars have in fact been observed^{6,7} with transverse velocities exceeding 300 km s^{-1} . New observations¹⁰ with the Very Large Array have found three radiopulsars with transverse velocities $> 670 \text{ km s}^{-1}$, and two other radiopulsars with velocities of $\sim 1,000 \text{ km s}^{-1}$ and $\sim 2,000 \text{ km s}^{-1}$ have also been discovered^{11,12}. The initial velocity distribution of neutron stars deduced from radiopulsar statistical analyses is very sensitive to the assumed luminosity evolution law and initial magnetic-field distribution¹³. A reasonably large population of high-velocity neutron stars could exist despite the low numbers of suitable radiopulsars observed because fast neutron stars quickly leave the plane of the Galaxy and fall below the detector threshold. Moreover, high velocities may be correlated with strong magnetic fields, which would imply shorter radiopulsar lifetimes because of the larger magneto-dipole energy losses from spinning, highly magnetized neutron stars¹⁴.

We examine whether a high-velocity population of neutron stars could account for the recent BATSE (Burst and Transient Source Experiment) observations of GRBs. Models involving high-velocity neutron stars for GRBs have been suggested^{4,15} before the BATSE results became available. Here we assume that the fast neutron stars produce most of the bursts, and that there is a specified delayed turn-on of the bursts which, for old neutron stars, approaches a constant bursting rate. In our calculations, we describe the spatial distribution of young stellar populations that produce neutron stars from supernovae using the model given by Paczyński⁴. We also use his galactic mass model with a cutoff radius of 70 kpc for the dark halo, giving an escape velocity from the centre of the Galaxy of $\sim 800 \text{ km s}^{-1}$ (even for a halo cutoff radius of 200 kpc, the escape velocity is only $\sim 900 \text{ km s}^{-1}$). For simplicity, we let all neutron stars in the high-velocity population be born with a randomly directed velocity of $1,000 \text{ km s}^{-1}$, which is added to the galactic rotation velocity at the birth site. We find that a broader velocity distribution does not significantly change the results as long as very few of the bursts originate from neutron stars with velocities

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$\leq 800 \text{ km s}^{-1}$. The neutron stars are assumed to be born uniformly in time during the past 10^{10} yr.

No GRBs have been associated with radiopulsars, so we assume that the GRB-active phase commences only after a neutron star passes through the 'death line' when radiopulsar activity ceases. This behaviour may result because filled radiopulsar magnetospheres short out bursting activity¹⁵. To quantify this effect, we propose a bursting rate function

$$\dot{N}_*(t) = K(1 - \exp\{-[t/\tau(B)]^\beta\}) \quad (1)$$

where $\tau(B)$ is the timescale during which a neutron star with surface polar magnetic field B is in the radiopulsar phase and does not burst. The proportionality constant K may be an extremely sensitive function of the neutron star birth parameters, such as the initial spin period or B . We also assume that neutron stars emit constant-luminosity bursts. The time dependence of the bursting behaviour at early times is determined by the

parameter β . We find that the results are insensitive to the precise form of the bursting rate function as long as $\dot{N}_*(t)$ displays the delayed turn-on behaviour.

Figure 1a shows the average dipole moment $\langle \cos \Theta \rangle$, quadrupole moment $\langle \sin^2 b \rangle$, and $\langle V/V_{\max} \rangle$ value, which characterizes spatial inhomogeneity, as a function of detector sampling distance r_d for neutron stars born with $1,000 \text{ km s}^{-1}$ velocities. Here we let $\tau = 30 \text{ Myr}$, and choose $\beta = 2$ in order to obtain $\langle V/V_{\max} \rangle \approx 0.5$ at small values of r_d (ref. 16). We also include neutron stars born from the Large Magellanic Cloud (LMC), Small Magellanic Cloud (SMC) and M31, which we weight by the factors 0.05, 0.0075 and 2, respectively, assuming that neutron stars are born at rates proportional to the number of stars in these galaxies compared with the Milky Way¹⁷. Because the plane of M31 is inclined by only $\sim 9^\circ$ with respect to the plane of our Galaxy¹⁸, we treat it as parallel. Inclination effects for the LMC and SMC are unimportant because of their relatively low masses. As can be seen, M31 contributes significantly to the burst statistics only when $r_d \geq 200 \text{ kpc}$, whereas the LMC and SMC have negligible effect.

The burst statistics are dominated by the outflowing neutron stars from our Galaxy if $r_d < 300 \text{ kpc}$. At $r_d \approx 100 \text{ kpc}$, $\langle \cos \Theta \rangle \approx 0.048$, $\langle \sin^2 b \rangle \approx 0.32$, and $\langle V/V_{\max} \rangle \approx 0.34$, with a statistical error $< 2\%$. These results are within 1.5σ of the BATSE values¹⁹ of $\langle \cos \Theta \rangle \approx 0.008 \pm 0.035$, $\langle \sin^2 b \rangle \approx 0.31 \pm 0.02$ and $\langle V/V_{\max} \rangle \approx 0.33 \pm 0.02$.

We also calculate burst statistics for the low-velocity population of neutron stars derived from the observed⁶ radiopulsar velocity distribution with no selection effects taken into account. The results (Fig. 1b) are in agreement with those of Paczyński⁴ at sampling distances $< 100 \text{ kpc}$, except for a small feature in $\langle V/V_{\max} \rangle$ near 55 kpc due to neutron stars from the LMC and SMC. As is well known, the low-velocity neutron stars cluster in a flattened distribution within $10\text{--}20 \text{ kpc}$ of our Galaxy and M31, giving burst statistics that are inconsistent with the BATSE results.

From Fig. 1a and b, we see that the fast population of neutron stars can explain the BATSE data only if the slow population contributes a very small percentage ($\leq 0.2\%$) of the bursts. We argue that the bursting properties of the two populations may in fact be very different, as is implied by the observed⁶⁻⁸ correlation between the magnetic moment and the velocity of radiopulsars. Recent analyses¹¹ also deduce this correlation and show that it cannot be solely explained by selection biases. If high-velocity neutron stars are born with magnetic fields $B \geq 5 \times 10^{12} \text{ G}$, then these neutron stars will have shorter radiopulsar lifetimes and longer periods during which they burst. With the standard dipole spindown rate, a neutron star with $B \leq 10^{12} \text{ G}$ will take $\geq 10^8 \text{ yr}$ to pass through the death line, and may thus rarely burst. The energy source for GRBs may originate from the available magnetic field energy, proportional to B^2 , which would favour the high-velocity neutron stars²⁰. There could also be a threshold field required to produce bursts. We also note that stronger magnetic fields in radiopulsars may be correlated with longer terminal rotation periods²¹, so that the low-velocity population may burst less frequently because short-period pulsars are rotationally stabilized against starquakes²². Finally, we reiterate that the ratio of high-velocity to low-velocity neutron stars may be much larger than observed if selection effects favour detection of the latter.

Figure 2 shows the $\log N(>F) - \log F$ distribution for our model compared with the BATSE data for 212 GRBs²² and the Pioneer Venus Orbiter (PVO) data²⁴ for 197 GRBs. $N(>F)$ is the number of bursts with peak flux greater than F . Because the BATSE size distribution is obtained from values of peak count rate for each burst, we do not provide an absolute flux scale. To normalize the BATSE and PVO data bases, which are uncertain to at least a factor of ~ 7 (ref. 24) we simply require a smooth transition connecting the two size distributions. Only the fast neutron stars were used in the model, and we obtain a

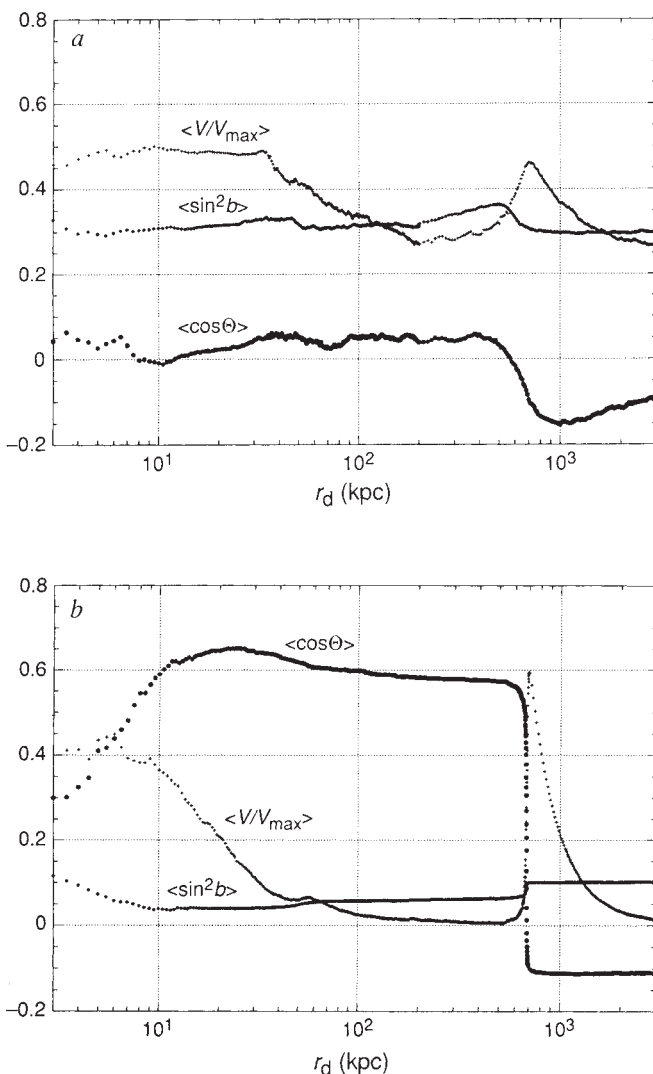


FIG. 1 a, Dipole moment $\langle \cos \Theta \rangle$, quadrupole moment $\langle \sin^2 b \rangle$, and $\langle V/V_{\max} \rangle$ values as a function of detector sampling distance r_d for high-velocity ($1,000 \text{ km s}^{-1}$) neutron stars. Θ is the angle between the burst and the Galactic Centre, and b is the galactic latitude of the burst. Here $\tau = 30 \text{ Myr}$ and $\beta = 2$. Good agreement with the BATSE data is obtained for $r_d \approx 100 \text{ kpc}$. The Monte Carlo simulation follows 15,000 neutron-star trajectories. b, Burst statistics as a function of r_d for neutron stars born with an initial velocity distribution derived from the observed^{6,7} radiopulsar velocity distribution (compare with a). The burst statistics are insensitive to the period t_y during which a neutron star is active as a burster, if $t_y \geq 50 \text{ Myr}$.

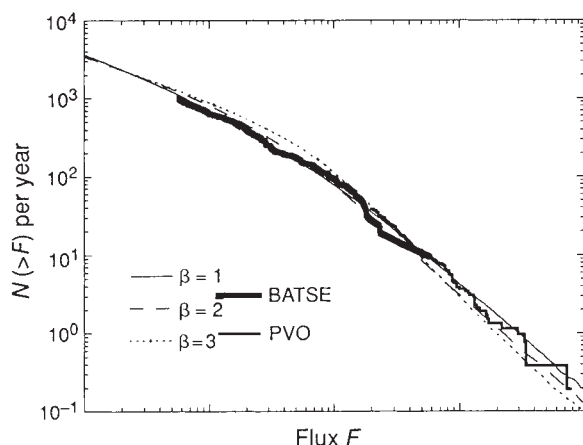


FIG. 2 Number of GRBs per year with peak flux $>F$ as a function of F . Thick curve shows the BATSE data²³ and the histogram shows the PVO data²⁴. The relative normalization of these two data sets is poorly known, and the size distribution of the BATSE data is derived from peak count rates of GRBs. Thus the absolute flux scale is uncertain. Curves show the model results with bursting function (1) for fast neutron stars characterized by the parameter β .

reasonable fit to the combined BATSE/PVO data with $\beta \approx 1-2$. We note, however, that our model does not require that the bright burst distribution follow a $-3/2$ power law. Thus the agreement of the PVO data⁵ with a homogeneous distribution of sources is, in the context of our model, fortuitous.

Our model provides a number of definite predictions which can be tested with the BATSE data as the statistics improve. In particular, the dipole moment for our fast neutron star model will be positive and the quadrupole moment will have a value $<1/3$, the isotropic value, at sampling distances $r_d \gg 10$ kpc because of the addition of the parallel rotation velocity to the isotropic ejection velocity of the newborn neutron stars (see Fig. 1a). We also estimate that two more years of data will be required to determine whether the brightest 2% of the bursts (flux $\geq 3 \times 10^{-5}$ erg cm⁻² s⁻¹) show a statistically significant spatial anisotropy inconsistent with an isotropic population. No anisotropy has been found, however, in analyses of bursts with fluxes in the range $10^{-6}-10^{-5}$ erg cm⁻² s⁻¹ (refs 25, 26).

The implied burst luminosity in our model is 10^{41-42} erg s⁻¹. If one fast neutron star is born every century in the Galaxy, there will be $\sim 10^6$ neutron stars within the 100 kpc sampling volume. A neutron star bursting roughly once every 1,000 yr will produce the observed burst rate, implying a total GRB energy budget of 10^{47-49} erg, depending on whether old ($\geq 10^8$ yr) neutron stars continue to burst. This amount of energy is not available in the magnetic field, but could be provided by the gravitational potential of the neutron star. The release of this energy could be dependent on the strength of the neutron star's field. GRBs with this luminosity could easily be detected by Rosat at X-ray energies from M31 (ref. 27), but because fast neutron stars occupy a much larger volume than a slow disc population, the rate of detection for the field-of-view of the Position Sensitive Proportional Counter on Rosat would be ≤ 0.2 per day. The detection rate of GRBs would be nearly uniform within 4° of M31. The inclusion of a GRB luminosity function does not significantly affect this result.

Our model for GRBs originating from galactic neutron stars is consistent with the BATSE and PVO results. This model is in accord with the concept that bursters originate from strongly magnetized, galactic neutron stars, deduced from years of GRB spectroscopy observations²⁷. We require a model where the faster neutron stars, which produce most of the bursts, escape from the gravitational potential of the Galaxy. The low- and high-velocity neutron stars differ greatly in their bursting properties, although the average burst luminosities may be similar

(compare ref. 29). In our model, bursts originate from neutron stars after the end of their radiopulsar phase. The concrete identification of the burst sources provides a considerable improvement over galactic GRB halo models in which the origin and spatial distribution of the sources is assumed from the outset^{30,31}. This model can be tested using improved burst statistics from BATSE, or by searching for bursters in the periphery of M31 using X-ray telescopes such as Rosat. $\square$

Received 19 March; accepted 24 August 1992.

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ACKNOWLEDGEMENTS. We thank R. J. Dufour, E. E. Fenimore, E. P. Liang and F. C. Michel for discussions and E. E. Fenimore for providing the PVO data.

Variable oxygen airglow on Venus as a probe of atmospheric dynamics

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THE venusian atmosphere exhibits two global-scale circulation patterns: an east-west super-rotation at altitudes above 100 km and below 70 km, and a solar-locked circulation between these heights, in which material rises on the dayside of the planet, flows around to the nightside and descends near the antisolar point (local midnight). The region at which these two flows interact (~ 95 km) is difficult to study, and existing data¹ are either in conflict or indicate long-term dynamical changes. Here we present high-resolution images of an oxygen airglow that arises during downwelling at ~ 95 km altitude on the nightside of Venus. Oxygen atoms are formed by photolysis of CO₂ on the sunlit hemisphere, and are then transported to the nightside, where they recombine during descent. The newly formed molecular oxygen emits radiation to produce the airglow. We observe localized, short-lived regions of emission and neighbouring dark areas, demonstrating

TABLE 1 Log of observations

Date 1991 UT	Zenith distance (degrees)	Apparent diameter (arcsec) (pixels)		Illuminated phase (%)	Radial velocity (km s ⁻¹)
July 27.35	69	44	56	18	-10.2
July 28.35	69	45	57	17	-10.0
September 17.02	41	45	57	17	9.1
September 19.85	61	44	56	18	9.6
September 19.90	49	44	56	18	9.6
September 20.85	62	43	54	19	9.8
September 20.91	47	43	54	19	9.8
October 17.79	67	30	38	41	12.4
October 18.79	68	29	37	41	12.4
October 19.79	68	29	37	42	12.4

the potential of such observations to constrain atmospheric dynamics at this little-studied and important altitude in the venusian mesosphere.

Free atomic oxygen is generated in the upper atmosphere of Venus when CO₂ is photolysed by sunlight. It is transported by the circulation cell² to the night hemisphere. Fox and Bougher³ have reviewed the chemistry and dynamics of this region, and show that the flow from the subsolar to the antisolar point crosses the terminator at altitudes in excess of 120 km (ref. 4).

As the gas descends, increasing atmospheric density encourages recombination from atomic to molecular oxygen. Direct recombination would violate spin selection rules, and more complex chain reactions are required. Many schemes have been proposed⁵, and several may operate, according to the availability of trace constituents that act as catalysts. Chemical models⁵ favour catalysts at altitudes near 94 km, with probable uncertainty of a few kilometres, deep within the downwelling region. The nocturnal downwelling is followed by return to the sunlit side at lower elevation, where the O₂ further combines with CO to form CO₂, completing the cycle. The entire flow is modified by the super-rotation of the upper atmosphere and by the interaction of thermal tides, so that the stagnation point feeding the downwelling normally lies on the morning side of the antisolar point. Figure 1 shows the cycle schematically.

Emission in the O₂ ¹Δ vibration-rotation band has been known for many years⁶. Previous spectroscopic observations covered large regions of the planet with poor spatial resolution. The band showed comparable intensity for the night and day hemispheres: 3–4 megaRayleighs (MR) before correction for reflection from underlying clouds (1 MR = 10¹² photons cm⁻² s⁻¹). The high intensity, almost 1,000 times greater than visible O₂ airglow bands⁷, requires that most of the oxygen atoms created by photolysis recombine and emit in the

1.27-μm band⁶. This result constrains recombination models. The 1.27-μm band closely monitors the distribution of all the downwelling atomic oxygen. It is of great interest also because it originates near the altitude where the subsolar-antisolar flow reverses, and between the two altitudes at which the thermal tides are driven⁸. Below and above this region there is strong super-rotation, whereas within it the super-rotation velocity may fall to zero, or be highly variable⁴.

Other, weaker airglow features, due to NO (refs 9, 10) and atomic oxygen^{11,12}, occur on the nightside of Venus and also represent gas transported from the sunlit hemisphere. The NO airglow is radiated at 115 ± 3 km altitude. It is variable and shows localized enhancements, usually near the equator at about 2 a.m. local (solar) time, with peak brightness about five times the hemisphere average¹⁰. Similar structure has been seen in the O I 130-nm emission¹³, which occurs at greater altitudes. The 1.27-μm O₂ airglow is formed several scale heights deeper than the NO.

Our O₂ images were made with the infrared camera/spectrograph IRIS¹⁴ on the 3.9-m Anglo-Australian Telescope (AAT). The detector is a 128 × 128 format HgCdTe array manufactured by Rockwell International. A spectroscopic resolution of $\lambda/\Delta\lambda \approx 400$, where λ is wavelength, is provided by a cross-dispersed transmission echelle¹⁵. A series of spectra is taken while the telescope is scanned back and forth normal to the slit direction. The slit was oriented east-west and we usually took three complete south-north-south double passes across the planet to average fluctuations caused by seeing. Each data set comprises two spatial and one spectral dimension from which monochromatic images may be extracted. This is a well established procedure at the AAT and has been used with other infrared and optical detectors. The images extended onto sky beyond both poles of the planet, but were restricted by the usable slit length to 12 arcsec east-west. To cover the dark hemisphere we therefore required several overlapping strips. The spatial resolution is determined by the slit width (1.36 arcsec), pixel width along the slit (0.79 arcsec) and sampling frequency in the cross-slit scan direction (0.8 arcsec).

Observations were made before inferior conjunction, in 1991 July, and again after the syzygy in September and October (Table 1). The July data were taken on a dark sky. The September observations were made against a daylight sky, and in October we observed minutes before sunrise. Standard spectroscopic reduction procedures were used for each frame in a data set. Scattered light dominates near the heavily overexposed sunlit portion of the planet, even though our images cover only one cusp of the crescent. To reveal detail there, we took the difference between images made on and off the O₂ emission. The proportion of the off-band images to be subtracted was deduced initially from the intensity of the sunlit crescent at the wavelengths of interest, and then refined by inspection. Because the crescent itself was saturated, the cusp may appear black or white on our subtracted images (for example, Fig. 2a). The reliability of intensities close to the sunlit crescent is lower than near the dark limb. Brightness variations of the sky during the scans,

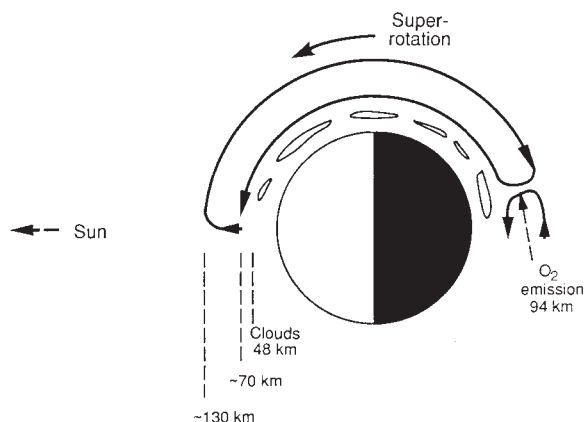


FIG. 1 Sectional sketch of the atmospheric dynamics on Venus. The O₂ emission arises where the subsolar-antisolar flow downwells, on the night-side. Approximate elevations above the mean surface are given. A similar cell on the lower side of the sketch has been omitted to permit labelling.

which are a problem with individual monochromatic images, become negligible when off-band images are subtracted.

Figure 2 shows the intensity distribution for several observing dates. The greyscale images have a common scale of surface brightness for ready comparison. The calibration was by observations of spectrophotometric standard stars, but is deemed no more precise than $\sim 50\%$ for two reasons. First, the observations were made at very high zenith distances where atmospheric absorption is difficult to correct. Second, saturation of the detector by the daylight sky prevented use of a wide slit to contain all the flux from the standard star. An additional uncertainty is telluric absorption in the same O_2 molecular band. Table 1 lists the radial velocity of the planet at each observation. High-resolution observations of the $1.27\text{-}\mu\text{m}$ band¹⁶ on Mars indicate that at the radial velocities used here the emission would be attenuated by less than 10%. We use observed intensities in the figures, and do not correct for the enhancement due to

reflection from the underlying clouds (approaching a factor of 3; compare with ref. 5) because of the lack of universally agreed correction. Nor do we correct for the thermal radiation from the lower atmosphere¹⁷ which contributes $\sim 10\%$ of the observed intensity.

The images reveal localized, often elongated regions of intense emission broadly distributed around the antisolar point, but sometimes seen at high latitudes or towards the terminator. Characteristic largest dimensions are 1,000 km. The features strongly resemble those seen in the NO airglow^{3,10}. An extended, diffuse background emission, including limb brightening, is also present but is not uniform across the planet. For example, in September a bright limb was present in only the northern hemisphere, whereas in October limb brightening was restricted to the southern hemisphere. Published NO airglow images have signal-to-noise ratios too low to indicate the distribution of the diffuse component, but again there is evidence of variable limb

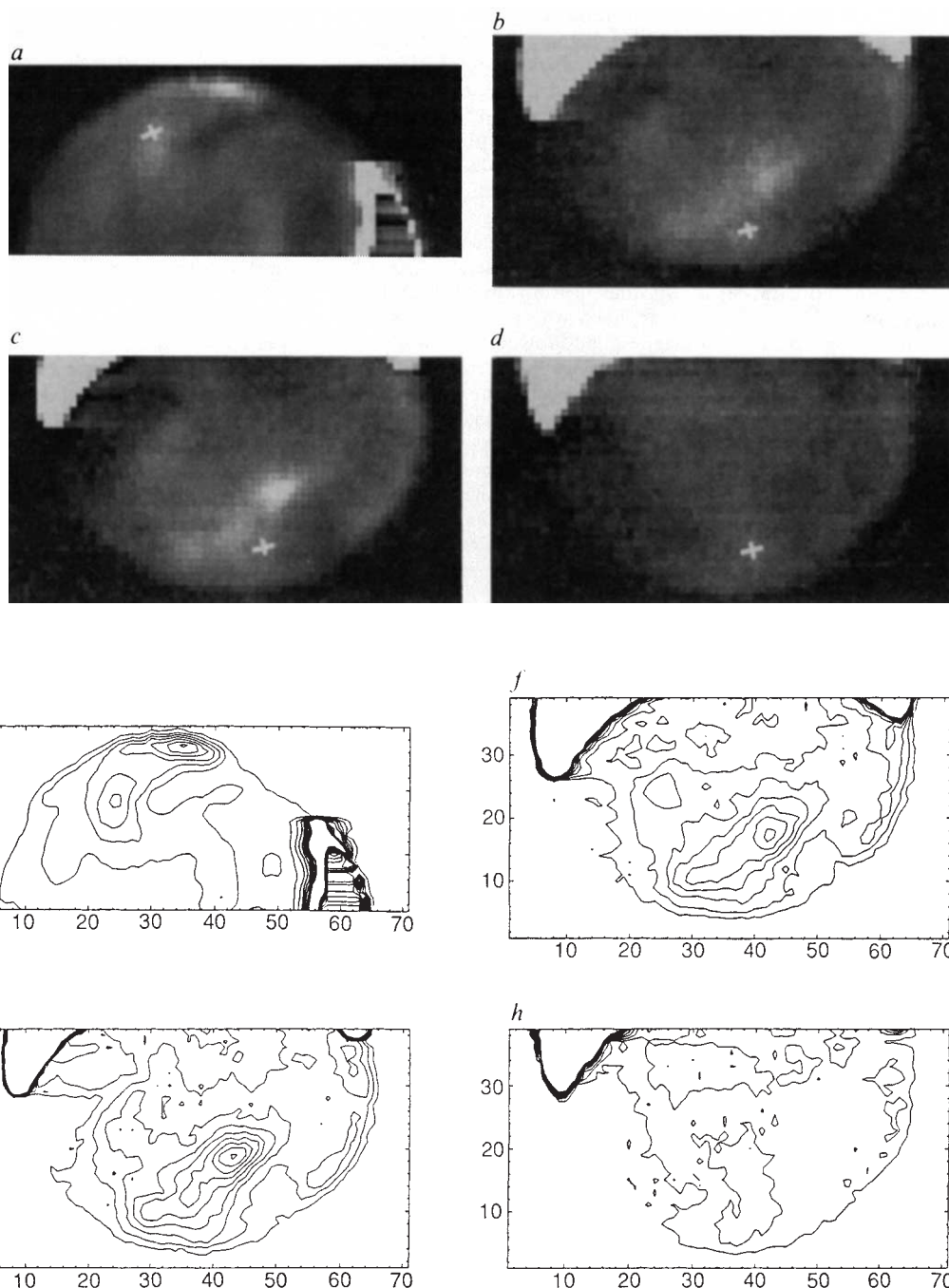


FIG. 2 Greyscale (*a-d*) and contour (*e-h*) images of Venus made on (*a, e*) July 27.35, (*b, f*) September 19.85, (*c, g*) September 19.90 and (*d, h*) September 20.85, after subtraction of off-band images to reduce scattered light. To show the dark features, the July image is reproduced at higher contrast than the other three. In all images north is to the right and a cross marks the antisolar point. The corresponding contour plots have axes in pixels and contours every 0.3 MR. The data have not been corrected for reflection from the underlying clouds. Emission in the $1.28\text{-}\mu\text{m}$ thermal window, ~ 0.3 MR, is included.

brightening¹⁰. Our images, in contrast, map large-scale patterns in downwelling. The limb brightening indicates that the emission is optically thin, as indeed is apparent from the line ratios observed at high spectral resolution⁶. We measure intensities only for the Q branch. Ignoring regions near the limb, the intensity of the brightest feature we see is comparable with the average intensity in 1975 over much of the dark side for the combined P, Q and R branches⁶.

The emission varies on a timescale of hours. On September 19 (Fig. 2f, g) the observed intensity of a bright region increased by more than 20% within 1.3 hours, from 1.9 to 2.4 MR after deducting 0.3 MR to correct for thermal emission from the deep atmosphere. On the following day (Fig. 2h) no corresponding bright feature was seen. Its maximum time constant was 20 hours. If we adopt the maximum downwelling velocity of 10 cm s^{-1} at the relevant altitudes^{4,18}, then the maximum vertical height over which emission arises within bright features is $\sim 7 \text{ km}$. This is considerably less than the $\sim 1,000\text{-km}$ horizontal scale. Models of the emission⁵ indicate a vertical range of 15–20 km for the reaction rate to lie within a factor e of the peak. The scale-height difference might indicate enhanced reaction rates due to density enhancement in the downwelling zone. Fox and Bougher³ argue, however, that none of the present atmospheric models is reliable enough for satisfactory comparison.

On the July image (Fig. 2a) several narrow, dark bands each traverse about 20 degrees of the planet close to the antisolar point. No similar feature has, to our knowledge, been recorded in other airglow lines. The intensity in these dark features seems to be determined entirely by the underlying thermal emission.

The absence of O_2 emission in the band is most readily interpreted as a lack of downwelling, which in turn suggests support by pockets of rising gas. A similar dark patch lies adjacent to the bright feature seen in September. If this interpretation is correct then we may be seeing the evidence for localized upwelling at $\sim 90 \text{ km}$ altitude.

Hourly observations of the localized O_2 features would monitor the winds, and might also reveal the vertical scale of the recombination regions. At the maximum downward velocity of $\sim 10 \text{ cm s}^{-1}$, the gas requires several days to descend from the NO to the O_2 level. Simultaneous series of observations of both airglow features may constrain models of the downwelling. $\square$

Received 22 May; accepted 2 September 1992.

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Determination of complex structures by combined neutron and synchrotron X-ray powder diffraction

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THE feasibility of determining crystal structures from powder diffraction data has improved substantially during the past decade. Early work using laboratory X-ray data^{1,2} has been followed by studies that take advantage of the higher resolution provided by synchrotron X-ray³ and neutron⁴ diffraction instrumentation. Other advances have been made in the computational aspects of the problem^{5,6}. Nevertheless, there has remained a disparity between the complexity of structures that can be solved, *ab initio*, from powder data, and those that can in principle be refined by the Rietveld profile method⁷. For example, refinements with up to 34 atoms⁸ and 132 positional parameters⁹ have been reported, but the most complex unknown structure to be solved from powder data contains only 17 atoms in the asymmetric unit cell¹⁰. Here we describe the solution and refinement of $\text{Ga}_2(\text{HPO}_3)_3 \cdot 4\text{H}_2\text{O}$, a novel framework structure with 29 atoms in the asymmetric unit

cell and 117 structural parameters, by the combined use of synchrotron X-ray and neutron powder diffraction. Exploiting the complementary nature of these techniques further extends the power of powder diffraction for structure determination.

The wide range of forms taken by microporous aluminosilicates, including zeolites, clays and pillared clays, is well known, but only recently has it become clear that aluminophosphates show similar versatility. A group of three-dimensional AlPO_4 structures were discovered^{11,12} in the early 1980s. These compounds have interesting sorption properties and can be rendered acidic by the partial replacement of phosphorus by silicon. Work on the analogous gallium phosphates led to the discovery of 'cloverite', a porous structure with an unprecedented 20-tetrahedral atom opening¹³. Our work involves phases containing the phosphite ion $(\text{HPO}_3)^{2-}$ rather than phosphate $(\text{PO}_4)^{3-}$. These are of interest because the terminal P–H groups may lead to materials in which the channels or cavities are lined by hydrogen, rather than oxygen as is found in zeolites and aluminophosphates. Framework structures of this type that have been prepared include the channel structures $\text{Zn}(\text{H}_2\text{PO}_3)_2 \cdot 3\text{H}_2\text{O}$ (ref. 14) and $\text{M}_2(\text{HPO}_3)_3$ (M is Fe, Al or Ga)^{15,16}.

We prepared the gallium phosphite sample by refluxing a solution of GaCl_3 and H_3PO_3 (molar ratio 1:1.7) in 100 ml of water for 24 h. The white polycrystalline product was deposited on the bottom of the flask and recovered by suction filtration. Preliminary X-ray data collected on a Scintag PAD X diffractometer indicated that the powder was of good crystallinity. The peak positions, indexed by the auto-indexing program TREOR¹⁷, indicated a monoclinic unit cell with lattice vectors $a = 8.13 \text{ \AA}$, $b = 10.02 \text{ \AA}$, $c = 7.70 \text{ \AA}$ and $\beta = 111.5^\circ$; the sample was apparently single-phase. Thermogravimetric analysis showed a weight loss of $\sim 15\%$ at a temperature of $\sim 180^\circ$, and ^{31}P magic-angle-spinning NMR indicated that there were three distinct phosphorus sites in the structure. On the basis of these analyses, we initially postulated that the composition of the compound was $\text{Ga}_2(\text{HPO}_3)_3 \cdot 3\text{H}_2\text{O}$.

Synchrotron X-ray diffraction data (Fig. 1a) were collected on beam line X7A at the National Synchrotron Light Source, Brookhaven National Laboratory. The sample was contained in a capillary, and data were collected over the angular range $6^\circ \leq 2\theta \leq 75^\circ$ using X-rays of mean wavelength 1.29915(2) Å. By inspecting the systematic absences in the powder pattern, and combining these with measurements which showed the compound to be roughly 1.5 times better than quartz at second-harmonic generation, the space group was assigned as $P2_1$. Integrated intensities were obtained by the Le Bail method¹⁸ using the GSAS¹⁹ suite of programs. Least-squares analysis of the profile included terms for the scale factor, background, lattice parameters, diffractometer zero point and peak shape (pseudo-Voigt). Unfortunately, as the capillary was static during data collection because of an instrumental malfunction, poor sampling of the powder led to systematic errors in the observed profile. The final profile agreement was therefore poor, but nevertheless, owing to the exceptional instrumental resolution, it proved possible to extract structure factors for 551 reflections from the profile. The structure factors were entered into the Oxford CRYSTALS²⁰ suite of programs, where 19 reflections were rejected as having zero intensity. The remaining 532 unique structure factors were then entered into SHELXS-86 (ref. 21), and a partial model for the structure was obtained. Structure solution involved the estimation of 151 phases for $|E|$ values greater than 1.2, and used 1,468 unique triplets and 168 negative quartets (SHELXS-86 combined figure of merit 0.117). This solution produced an E -map with two large peaks, which were assigned as gallium atoms, and subsequent Fourier syntheses revealed four more atoms which could be assigned as two phosphorus and two oxygen atoms. This model was then entered into GSAS, and Rietveld refinement, coupled with further Fourier syntheses, revealed the positions of one further phosphorus atom and 10 more oxygen atoms, giving a total of 17 atoms in the asymmetric unit.

To improve the overall quality of the structure, and to determine the locations of the hydrogen atoms, we collected neutron diffraction data at the National Institute of Standards and Technology using diffractometer BT-1. A 9-g sample of the compound was prepared from deuterated water, and data were collected at 8.7 K, using neutrons of mean wavelength 1.5454(1) Å, in the range $10^\circ \leq 2\theta \leq 120^\circ$. The model obtained from the refinement against the synchrotron X-ray data was used as a starting point for the analysis of the neutron data. Using computer graphics techniques, we added all the phosphite deuterium atoms to the model, and by close inspection of the possible hydrogen bonding

TABLE 1 Fractional coordinates and U_{iso} (Å²) for Ga₂(HPO₃)₃·4H₂O

	x	y	z	U_{iso}
Ga(1)	0.0226 (10)	0.3523 (7)	0.0645 (11)	0.01‡
Ga(2)	0.3766 (9)	0.0757 (7)	0.5854 (11)	0.01‡
P(1)	0.3439 (12)	0.3714 (12)	0.4556 (13)	0.01‡
P(2)	-0.0103 (12)	0.0776 (11)	0.2485 (14)	0.01‡
P(3)	-0.0859 (14)	0.6313 (11)	0.2189 (15)	0.01‡
O(1)	0.2586 (12)	0.3682 (11)	0.2396 (14)	0.006 (2)
O(2)	-0.0497 (13)	0.4829 (10)	0.2035 (14)	0.003 (2)
O(3)	-0.0568 (14)	0.2223 (10)	0.2059 (15)	0.002 (2)
O(4)	0.1242 (12)	0.2071 (10)	-0.0378 (15)	0.001 (2)
O(5)	0.0906 (14)	0.4827 (9)	-0.0849 (16)	0.011 (2)
Ow(6)	-0.2140 (15)	0.3406 (10)	-0.1256 (13)	0.016 (2)
O(7)	0.4555 (13)	0.2595 (10)	0.5453 (14)	0.013 (2)
O(8)	0.1954 (13)	0.0480 (10)	0.3444 (14)	0.002 (2)
O(9)	-0.2162 (12)	0.6551 (10)	0.3096 (13)	0.004 (2)
O(10)	0.5391 (13)	0.0007 (9)	0.4861 (14)	0.008 (2)
Ow(11)	0.5698 (14)	0.0915 (10)	0.8478 (15)	0.008 (2)
Ow(12)	0.3224 (12)	-0.1031 (9)	0.6751 (13)	0.009 (2)
Ow(13)	0.4925 (15)	0.1676 (11)	0.1586 (16)	0.011 (2)
Dp(1)	0.2089 (11)	0.3938 (8)	0.5259 (11)	0.005 (2)
Dp(2)	-0.0860 (13)	0.0425 (10)	0.3821 (14)	0.011 (2)
Dp(3)	0.0843 (14)	0.6729 (12)	0.3454 (14)	0.026 (3)
Dw(61)	-0.2550 (15)	0.4054 (13)	-0.2428 (16)	0.036 (3)
Dw(62)	0.3010 (16)	0.7752 (11)	0.1647 (16)	0.042 (3)
Dw(111)	0.6701 (19)	0.0309 (14)	0.8855 (19)	0.053 (4)
Dw(112)	0.5376 (15)	0.1131 (11)	0.9477 (16)	0.033 (4)
Dw(121)	0.4005 (15)	-0.1797 (11)	0.7265 (17)	0.032 (3)
Dw(122)	0.2216 (13)	-0.1039 (11)	0.7159 (14)	0.023 (3)
Dw(131)	0.3614 (17)	0.1900 (14)	0.1081 (18)	0.033 (4)
Dw(132)	0.5187 (20)	0.1127 (14)	0.2653 (14)	0.052 (5)

The crystallographic space group is $P2_1$ with lattice parameters $a = 8.0947$ (2) Å, $b = 10.0336$ (2) Å, $c = 7.6711$ (2) Å and $\beta = 111.392$ (2)°. U_{iso} is the isotropic temperature factor.

‡ Values fixed.

distances in the structure, we obtained the positions of two of the water deuteriums. This new model was sufficiently close to the correct one for successful Rietveld refinement against the neutron data, and an additional water oxygen and the remaining deuterium atoms were located from subsequent cycles of refinement and Fourier synthesis. At this stage, the composition of the new phase was finally confirmed as Ga₂(HPO₃)₃·4H₂O, rather than Ga₂(HPO₃)₃·3H₂O. When the 'extra' water molecule was included in the model for refinement against the synchrotron X-ray data, the fitting of the profile improved, thus confirming

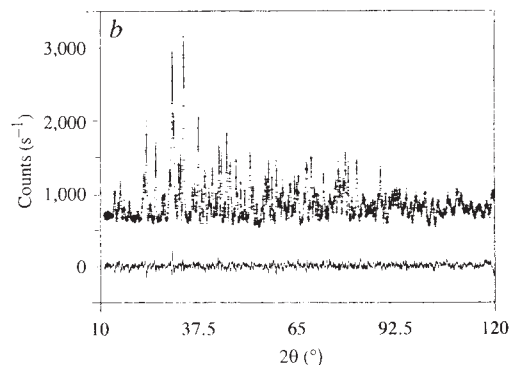
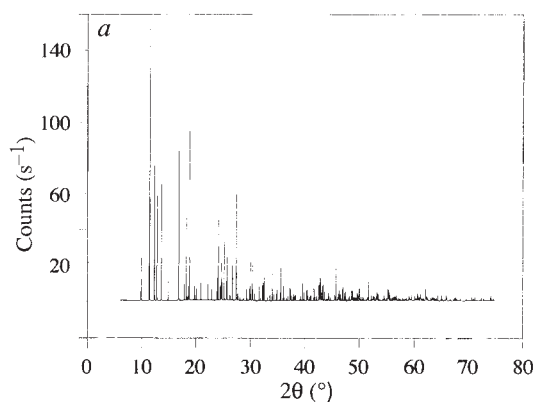


FIG. 1 a, Synchrotron powder pattern for Ga₂(HPO₃)₃·4H₂O obtained using beam line X7A at the National Synchrotron Light Source. The data were collected with X-rays of wavelength $\lambda = 1.29915(2)$ Å, using a Si(111) channel cut monochromator and Ge(220) analysing crystal. The pattern consists of 6,903 data points. b, Final observed, calculated and difference profile plots for the neutron refinement of Ga₂(HPO₃)₃·4H₂O. The data were collected on beamline BT-1 at the National Institute of Science and Technology, using

neutrons of wavelength 1.5454(1) Å selected using a Cu(220) monochromator. The final cycle of least-squares refinement contained terms for 138 variables and converged to final agreement factors of $R_F = 2.91\%$, $R_p = 3.49\%$, $R_{wp} = 4.39\%$ and $\chi^2 = 1.77$ (see ref. 19 for description of the R factors). The peak shape was fitted using a gaussian function, with full width at half maximum parameters $U = 673(28)$, $V = -653(31)$, $W = 285(8)$.

TABLE 2 Selected bond distances and angles for $\text{Ga}_2(\text{HPO}_3)_3 \cdot 4\text{H}_2\text{O}$

Bond distances					
Ga(1)–O(1)	1.899 (12)	Ga(2)–O(7)	2.011 (12)	P(3)–O(2)	1.530 (15)
Ga(1)–O(2)	1.912 (13)	Ga(2)–O(8)	1.915 (12)	P(3)–O(2)	1.514 (14)
Ga(1)–O(3)	1.949 (12)	Ga(2)–O(9)	1.932 (11)	P(3)–O(9)	1.479 (14)
Ga(1)–O(4)	1.971 (13)	Ga(2)–O(10)	1.899 (12)	P(3)–Dp(3)	1.428 (14)
Ga(1)–O(5)	1.946 (12)	Ga(2)–Ow(11)	2.056 (13)		
Ga(1)–Ow(6)	1.942 (14)	Ga(2)–Ow(12)	2.027 (12)		
P(2)–O(3)	1.505 (15)	P(1)–O(1)	1.546 (13)		
P(2)–O(5)	1.520 (14)	P(1)–O(7)	1.448 (14)		
P(2)–O(8)	1.583 (14)	P(1)–O(10)	1.572 (15)		
P(2)–Dp(2)	1.418 (15)	P(1)–Dp(1)	1.402 (11)		
Ow(6)–Dw(61)	1.060 (14)	Ow(11)–Dw(111)	0.970 (15)		
Ow(6)–Dw(62)	0.929 (13)	Ow(11)–Dw(112)	0.921 (14)		
Ow(12)–Dw(121)	0.981 (12)	Ow(13)–Dw(131)	1.013 (15)		
Ow(12)–Dw(122)	0.975 (13)	Ow(13)–Dw(132)	0.944 (17)		
Bond angles					
O(1)–Ga(1)–O(2)	88.9 (5)	Ow(11)–Ga(2)–O(7)	84.7 (5)		
O(1)–Ga(1)–O(3)	96.5 (6)	Ow(11)–Ga(2)–O(8)	176.0 (6)		
O(1)–Ga(1)–O(4)	83.4 (6)	Ow(11)–Ga(2)–Ow(12)	85.2 (5)		
O(1)–Ga(1)–Ow(6)	176.6 (7)	Ow(11)–Ga(2)–O(9)	86.9 (5)		
O(1)–Ga(1)–O(5)	86.1 (5)	Ow(11)–Ga(2)–O(10)	91.9 (5)		
O(2)–Ga(1)–O(3)	85.3 (5)	O(7)–Ga(2)–O(8)	99.2 (6)		
O(2)–Ga(1)–O(4)	170.4 (6)	O(7)–Ga(2)–Ow(12)	169.7 (7)		
O(2)–Ga(1)–Ow(6)	92.4 (5)	O(7)–Ga(2)–O(9)	89.1 (5)		
O(2)–Ga(1)–O(5)	94.4 (5)	O(7)–Ga(2)–O(10)	89.9 (5)		
O(3)–Ga(1)–O(4)	89.8 (5)	O(8)–Ga(2)–Ow(12)	90.8 (6)		
O(3)–Ga(1)–Ow(6)	86.7 (6)	O(8)–Ga(2)–O(9)	93.9 (5)		
O(3)–Ga(1)–O(5)	177.3 (7)	O(8)–Ga(2)–O(10)	87.4 (5)		
O(4)–Ga(1)–Ow(6)	95.6 (5)	Ow(12)–Ga(2)–O(9)	87.8 (5)		
O(4)–Ga(1)–O(5)	90.8 (5)	Ow(12)–Ga(2)–O(10)	93.0 (5)		
Ow(6)–Ga(1)–O(5)	90.7 (6)	O(9)–Ga(2)–O(10)	178.5 (6)		
O(1)–P(1)–O(7)	116.8 (10)	O(2)–P(2)–O(8)	114.8 (9)		
O(1)–P(1)–O(10)	108.0 (8)	O(3)–P(2)–O(5)	115.3 (9)		
O(1)–P(1)–Dp(1)	108.0 (8)	O(3)–P(2)–Dp(2)	104.8 (10)		
O(7)–P(1)–O(10)	107.4 (8)	O(8)–P(2)–O(5)	109.1 (8)		
O(7)–P(1)–Dp(1)	112.3 (8)	O(8)–P(2)–Dp(2)	106.0 (8)		
O(10)–P(1)–Dp(1)	103.4 (8)	O(5)–P(2)–Dp(2)	105.9 (9)		
O(2)–P(3)–O(4)	113.5 (9)				
O(2)–P(3)–O(9)	112.5 (9)				
O(2)–P(3)–Dp(3)	99.9 (9)				
O(4)–P(3)–O(9)	114.1 (9)				
O(4)–P(3)–Dp(3)	107.5 (9)				
O(9)–P(3)–Dp(3)	108.0 (10)				
Dw(61)–Ow(6)–Dw(62)	101.2 (14)	Dw(111)–Ow(11)–Dw(112)	111.0 (15)		
Dw(121)–Ow(12)–Dw(122)	110.9 (14)	Dw(131)–Ow(13)–Dw(132)	109.5 (17)		

Bond distances in Å, bond angles in degrees.

that the two samples were of the same composition. The final Rietveld analysis of the neutron data for $\text{Ga}_2(\text{HPO}_3)_3 \cdot 4\text{H}_2\text{O}$, with 29 atoms in the asymmetric unit, is shown in Fig. 1b, and the final structural parameters, and selected bond lengths and angles, are given in Tables 1 and 2, respectively. Both the quality of the profile fit and the chemical acceptability of the bond lengths and angles attest to the correctness of the final model.

The phosphite anions in $\text{Ga}_2(\text{HPO}_3)_3 \cdot 4\text{H}_2\text{O}$ show the usual tetrahedral arrangement of three oxygens and one hydrogen about the phosphorus atom; the bond distances and angles are comparable with those obtained from single-crystal X-ray studies on similar compounds^{14–16}. The gallium atoms show almost regular coordination by oxygen, Ga(1) being bonded to five phosphite oxygens and a water molecule, and the Ga(2) polyhedron comprising four phosphite oxygens and two waters. The remaining water molecule (Ow13) is held in a small cavity (diameter ~ 5.5 Å) by hydrogen bonding.

The structure (Fig. 2) is made up of units comprising the two GaO_6 octahedra linked by three phosphite groups through shared oxygen atoms. These units, which are also seen in other materials of this kind, including $\text{Fe}_2(\text{HPO}_3)_3$ (ref. 15), $\text{M}_2(\text{HPO}_3)_3$ where M is Al or Ga (ref. 16), and $\text{Al}_2(\text{SeO}_3)_3 \cdot x\text{H}_2\text{O}$ ($x=3$ or 6) (refs 22, 23), are connected to form a three-dimensional framework by the sharing of oxygens with other

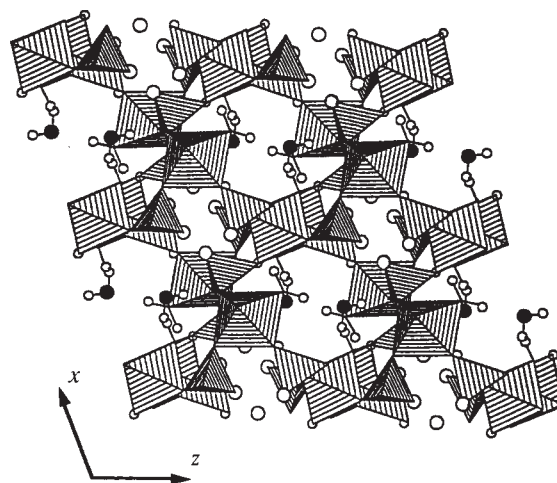


FIG. 2 Polyhedral representation of the structure of $\text{Ga}_2(\text{HPO}_3)_3 \cdot 4\text{H}_2\text{O}$ viewed parallel to the [010] direction. The figure depicts gallium-centred octahedra and phosphorus-centred tetrahedra. Hydrogen atoms are shown as open spheres and Ow(13) is shown as a closed sphere.

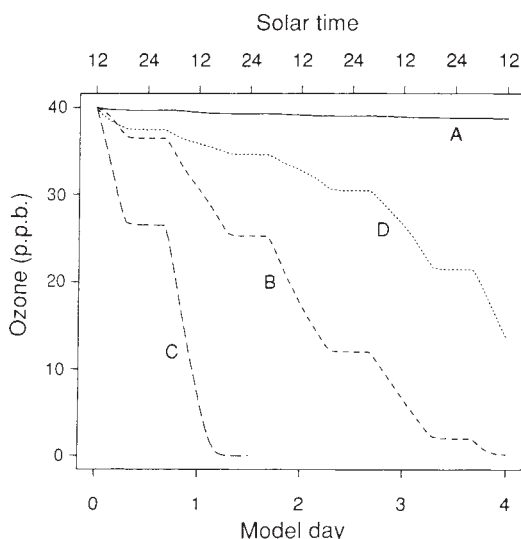
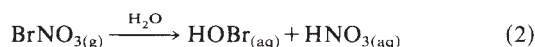


FIG. 2 Ozone losses computed in the photochemical model for a surface-air parcel in Arctic spring (82.5° N, 1 April). The initial ozone concentration in the air parcel is 40 p.p.b.v. Simulation A includes gas-phase chemistry only, with initial conditions 50 p.p.t.v. inorganic bromine (Br_x) and 50 p.p.t.v. NO_x . Simulations B–D include aerosol reactions (1) and (2), with initial conditions: B, 50 p.p.t.v. Br_x and 50 p.p.t.v. NO_x ; C, 100 p.p.t.v. Br_x and 50 p.p.t.v. NO_x ; D, 50 p.p.t.v. Br_x and 1 p.p.t.v. NO_x .

forward reaction (1), calculated from the above data and assuming phase equilibrium for the nonlimiting reactant (either HOBr or HBr), is several orders of magnitude shorter than the characteristic time for gas-phase diffusion of the limiting reactant to the aerosol surfaces ($\sim 10^3$ s; ref. 15). We conclude that the production of $\text{Br}_{2(\text{aq})}$ by reaction (1) is limited by the rate of uptake of either HBr or HOBr by the aerosol. Ionic strength corrections could not be large enough to modify our conclusion. Data for the analogous reaction involving Cl species in concentrated sulphuric acid indicate that there is little effect of ionic strength on reaction rate^{9,14}.

As $\text{Br}_{2(\text{aq})}$ is produced it volatilizes to the gas phase; the Henry's law constant is low, 20 M atm⁻¹ at 240 K (ref. 16). It can be inferred that hydrolysis of $\text{Br}_{2(\text{aq})}$ by the reverse reaction (1) is too slow to compete with volatilization; the characteristic time for molecular diffusion out of typical 1- μm -diameter aerosol particles is $\sim 10^{-4}$ s (ref. 15). The gas-phase Br_2 is rapidly photolysed during daytime (time constant ~ 1 min), but it may accumulate at night.

Reaction (1) also provides a means for recycling BrNO_3 produced in the gas phase by the reaction $\text{BrO} + \text{NO}_2$ (Fig. 1). We expect that BrNO_3 should be rapidly scavenged by the aerosol and hydrolysed to HOBr, by analogy with data for ClNO_3 (ref. 14)



followed by subsequent reaction of $\text{HOBr}_{(\text{aq})}$ and Br^- by (1) to produce Br_2 .

We examined the contributions of reactions (1) and (2) to ozone depletion in the Arctic using a photochemical box model¹⁷ applied to a surface-air parcel at Alert, Canada (82.5° N). The model was initialized at the onset of an ozone depletion event with the following air composition taken from typical measurements at Alert³: 40 parts per 10⁹ (p.p.b.v.) ozone, 50 p.p.t.v. NO_x ($\text{NO} + \text{NO}_2$), 100 p.p.b.v. CO, 40 p.p.t.v. formaldehyde, 60 p.p.t.v. acetaldehyde, and 50 p.p.t.v. Br_x assumed to be present initially as BrO. The chemical evolution of the air parcel was computed for several model days, starting at noon, using kinetic and photochemical data from recent compilations^{8,18–20}.

The rate of reaction (1) was computed as the gas-phase diffusion flux²¹ of the limiting species (HOBr, BrNO_3 or HBr) to a sulphate aerosol with a size distribution taken from mean observations for April, at the site¹⁰. The rate of reaction (2) was computed as the gas-phase diffusion flux of BrNO_3 to the aerosol. The diffusion flux depends in part on the sticking probability, α , of the gas at the aerosol surface. We estimate $\alpha = 0.01$ for HOBr and BrNO_3 , and $\alpha = 0.3$ for HBr, on the basis of data for the corresponding Cl species over water/ H_2SO_4 solutions^{14,22}; these estimates are probably conservative. The resulting time-constants for gas uptake by the aerosol are 7×10^3 s for HOBr, 8×10^3 s for BrNO_3 , and 5×10^2 s for HBr. Photon intensities were calculated for clear-sky conditions on 1 April; the 8-h twilight on 1 April was treated in the model as darkness. A fixed temperature of 240 K and a relative humidity of 75% were assumed.

The computed ozone losses are shown in Fig. 2. When only gas-phase chemistry is considered (simulation A), HBr sequesters the bromine, and ozone depletion is only a few per cent after four model days. In contrast, near-total ozone depletion is rapidly achieved when reactions (1) and (2) are considered; the depletion requires a few days with 50 p.p.t.v. Br_x (simulation B) and less than a day with 100 p.p.t.v. Br_x (simulation C). We find in simulations B and C that BrO dominates the bromine pool in the daytime (Fig. 3) because of rapid regeneration of Br and BrO radicals through reaction (1). At night BrO is depleted by a minor branch of the self-reaction producing Br_2 (Fig. 1), and Br_2 becomes the dominant bromine species. Filterable bromine was found to be formed when nighttime air from Barrow (Alaska) was exposed to artificial solar radiation⁵; this would be consistent with our mechanism if BrO were collected as filterable bromine. We predict here that little Br_x should remain on the aerosol throughout rapid ozone depletion events; a light-sensitive source of Br_2 (ref. 7) would cause Br_x to be present mostly on the aerosol at night.

The regeneration of bromine radicals by reaction (1) is contingent on a sustained supply of HBr and HOBr. We found in simulations B and C that the rate of reaction (1) is limited during the first two days by the supply of HOBr and that HBr becomes limiting later as the aldehydes are depleted. Near-total loss of NO_x takes place during the first day of simulation, reflecting the rapid formation of BrNO_3 followed by reaction (2) producing HNO_3 . This result is consistent with observations indicating low concentrations of NO_x during ozone depletion events³.

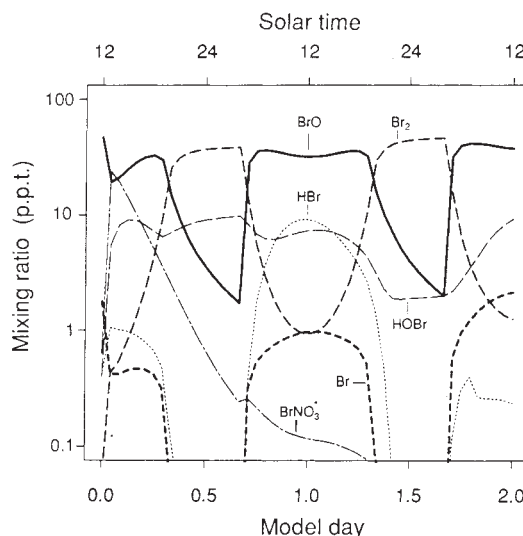


FIG. 3 Partitioning of inorganic bromine species in simulation B, for the first two days of simulation.

Sensitivity calculations for the air parcel with 50 p.p.t.v. Br_x (simulation B) show that ozone depletion can take place over a wide range of model conditions. A calculation with low initial NO_x (1 p.p.t.v.) indicates slow ozone loss during the first two days, because HOBr is not supplied by BrNO₃ hydrolysis, but the loss rate increases after the second day as depletion of the aldehydes moderates the source of HBr (simulation D; Fig. 3). Absence of aldehydes in the initial atmosphere has only a small effect on computed ozone loss because the reaction Br+HO₂ sustains a source of HBr that allows reaction (1) to proceed. Decreasing the sticking coefficients of HOBr and BrNO₃ from 0.01 to 0.001 slows down ozone loss, but near-total ozone depletion is still achieved within a week. Changing the HOBr photolysis rate constant by a factor of two, to reflect current uncertainty^{4,19}, does not affect results significantly.

Improved field data for the speciation of bromine during ozone depletion episodes are evidently needed to evaluate our hypothesis. Measurements of the BrO concentration would in particular allow a direct assessment of the rate of bromine-catalysed ozone loss. Measurements of the Br₂ concentration at night would allow us to distinguish between a light-sensitive source of Br₂ (resulting in night-time Br_x being present mostly as aerosol⁷) and a light-insensitive source, as proposed here. □

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Received 11 May; accepted 17 August 1991.

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ACKNOWLEDGEMENTS. This work was supported by the NSF and by the Packard Foundation. We thank S. C. Wofsy, N. D. Sze and S. M. Li for useful discussions.

Flattening of the sea-floor depth-age curve as a response to asthenospheric flow

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THE flattening of sea-floor depths from the $\sqrt{\text{age}}$ dependence predicted by considering the cooling plate as a growing thermal boundary layer is a fundamental constraint on the evolution of oceanic lithosphere¹. Previous explanations for the flattening have included reheating from convective instabilities that form beneath lithosphere older than ~80 Myr (ref. 2), thermal rejuvenation of lithosphere that passes over stationary hotspots^{3–5}, or a whole-mantle flow forced by two converging plates⁶. We suggest here that the flattening of old ocean floors can perhaps best be explained as a dynamic phenomenon reflecting flow in asthenosphere underlying the oceanic lithosphere. Applying the model to the Pacific plate, we show that a solution for flow in an asthenosphere low-viscosity channel which is 'consumed' by plate accretion and the subduction of lithosphere at trenches, and replenished by near-ridge upwelling and near-ridge hotspots, generates the observed ~1 km of sea-floor flattening for asthenospheric viscosities of 2×10^{18} Pa s and an absolute Pacific plate motion of 100 mm yr⁻¹. Our model can also explain the asymmetric subsidence of the South American and African plates away from the Mid-Atlantic Ridge.

We start from a feeling that previous explanations for the flattening of the sea-floor depth-age curve are not entirely satisfactory. Hotspot rejuvenation models^{3,4} predict heat flow and seismic slowness anomalies, yet no such anomalies are observed^{7–9}. The classic 'plate' model¹ assumption of small-scale convective instabilities² is not supported by recent analyses of depth^{10,11}: the flattening occurs at different ages in different regions, and in some is not observed at all¹⁰. The conclusion⁵ that large-scale mantle-flow induced reheating can explain the flattening is based on numerical experiments, the conclusions of which are strongly affected by the assumed two-dimensional geometry, with cylindrical sheets of upwelling instead of the observed point-like (hotspot) upwelling structures.

To see the plausible dynamic effects of asthenosphere flow, consider the following idealization. Imagine the asthenosphere to be a low-viscosity channel bounded at its top by a high-viscosity lithosphere (defined by an isotherm) and at its base by a high-viscosity mesosphere (defined by an isobar). Flow within the asthenosphere is determined by the shear between plate motions and underlying slower mesospheric flow, the incorporation (accretion) of asthenosphere into the ageing and growing lithosphere, and preferential asthenospheric replenishment at hotspots. Because the asthenosphere is much thinner than it is wide, to a first approximation we can treat flow within the asthenosphere with standard lubrication-theory approximations developed for viscous flow in a narrow channel¹², as done in previous studies of asthenospheric return flow^{13–16}. Unlike previous studies, however, we incorporate one further physical constraint into our model problem: we explicitly consider the effects of a lithosphere that grows owing to plate cooling with age. This determines the rate at which asthenosphere is consumed and also determines the channel height or effective resistance to asthenospheric flow. The two types of material flow in this system (Fig. 1a) are (1) 'passive' flow due to motion of the lithosphere and asthenosphere shear between the fast-moving lithosphere and slower moving mesosphere, q_s ; and (2) 'dynamic' or pressure-driven asthenospheric flow, q_p , to bring asthenosphere to where it is 'consumed' by accretion into the lithosphere (Fig. 1a) and to match constraints on the net asthenosphere flux at a continental margin (Fig. 1b) or subduction zone (Fig. 1c).

For this model problem we make the rough approximation that all asthenosphere replenishment will occur near the ridge axis. (Morgan's original ensemble of large hotspots¹⁷ is statistically clumped near ridges¹⁸, although later compilations that include larger numbers of 'small' hotspots are not^{18,19}). Because the asthenosphere channel height is largest and typical asthenospheric viscosities lowest near a ridge axis, we consider that asthenospheric flow is channelled predominantly along a ridge axis where the resistance to asthenospheric flow is least and thus that flow into the 'ridge' boundary of this model problem comes from the out-of-plane direction. The complexities of the 'out-of-plane' ridge axis and closeness to the hotspot strictly require a two-dimensional numerical treatment²⁰, but the essential physics of realistic asthenospheric flow beneath old lithosphere is captured by this approximation, which has the virtue of leading to a simple closed-form solution. For the

model problem the net lithosphere plus asthenosphere flow through each horizontal column is constant: $q_p + q_s = Q_{\text{tot}}$, where the vertically integrated shear flow (Fig. 1a) $q_s(x) = U_a(h_0 - h(x)) + \frac{1}{2}U_a h(x)$ for an asthenosphere channel thickness $h(x)$, ridge channel thickness $h_0 = 200$ km, lithosphere thickness $(h_0 - h(x))$, absolute plate spreading rate U_a , and an assumed horizontal motion of the mesosphere $U_{\text{mesosphere}} = 0$. If we now consider the net flow within the asthenosphere shown in Fig. 1a, changes in $q_s(x)$ must be balanced by changes in the vertically integrated pressure flow $q_p(x)$ so that if we know the net horizontal flux Q_{tot} then $q_p(x) = Q_{\text{tot}} - q_s(x)$.

We now consider horizontal momentum and mass conservation in the asthenosphere channel. The uniform viscosity (μ) momentum equation becomes in the lubrication approximation

$$\mu \frac{\partial^2 u}{\partial z^2} = \frac{\partial P}{\partial x}$$

where u is velocity and P is pressure. This is integrated for the pressure-driven flux $q_p = \int_0^{h(x)} u_p(z) dz$

$$q_p = -\frac{h^3(x)}{12\mu} \frac{\partial P}{\partial x}$$

Integrating

$$\frac{\partial P}{\partial x} = -\frac{12\mu}{h^3(x)} (Q_{\text{tot}} - q_s(x))$$

leads to the horizontal pressure variation as a function of x . We

choose a lithosphere whose thickness increases as $St(x)^{1/2} = S\sqrt{x}/U_r$, where t is age, U_r maps plate age into distance x and S is a constant of proportionality with units of $\text{m s}^{-1/2}$. Within this parameterization

$$\frac{\partial P}{\partial x} = -\frac{12\mu}{(h_0 - (S/\sqrt{U_r})\sqrt{x})^3} \times \left(Q_{\text{tot}} - U_a \frac{h_0}{2} - \frac{U_a (S/\sqrt{U_r})\sqrt{x}}{2} \right)$$

This, integrated for dynamic pressure, yields

$$P(x) - P(0) = \gamma [\ln(\alpha(x)) + \alpha(x)^{-1} - 1 + (1 - (Q_{\text{tot}}/U h_0))(2\alpha(x)^{-1} - \alpha(x)^{-2} - 1)]$$

where $\gamma = 12\mu U_a U_r / S^2$ and $\alpha(x) = 1 - (S/h_0)\sqrt{x}/U_r$. Expressed in terms of the dynamic pressure topography $w = P(x)/(\rho_m - \rho_w)g$, where ρ_m and ρ_w are the densities of mantle and water, and g is the acceleration due to gravity

$$w(x) - w(0) = T_0 [\ln(\alpha(x)) + \alpha(x)^{-1} - 1 + (1 - Q_{\text{tot}}/U h_0)(2\alpha(x)^{-1} - \alpha(x)^{-2} - 1)]$$

where the dynamic topography magnitude

$$T_0 = \gamma / [(\rho_m - \rho_w)g] = 12\mu U_a U_r / [(\rho_m - \rho_w)g S^2]$$

A choice of $\mu = 2 \times 10^{18}$ Pa s, $U_a = U_r = 100$ mm yr $^{-1}$, and $S = \sqrt{\pi} 10^{-3}$ m s $^{-1/2}$ (this S yields a 100-km-thick lithosphere after 100 Myr) leads to $T_0 = 3.33$ km, and $\alpha(x) = (1 - 1.58 \times 10^{-4}\sqrt{x})$.

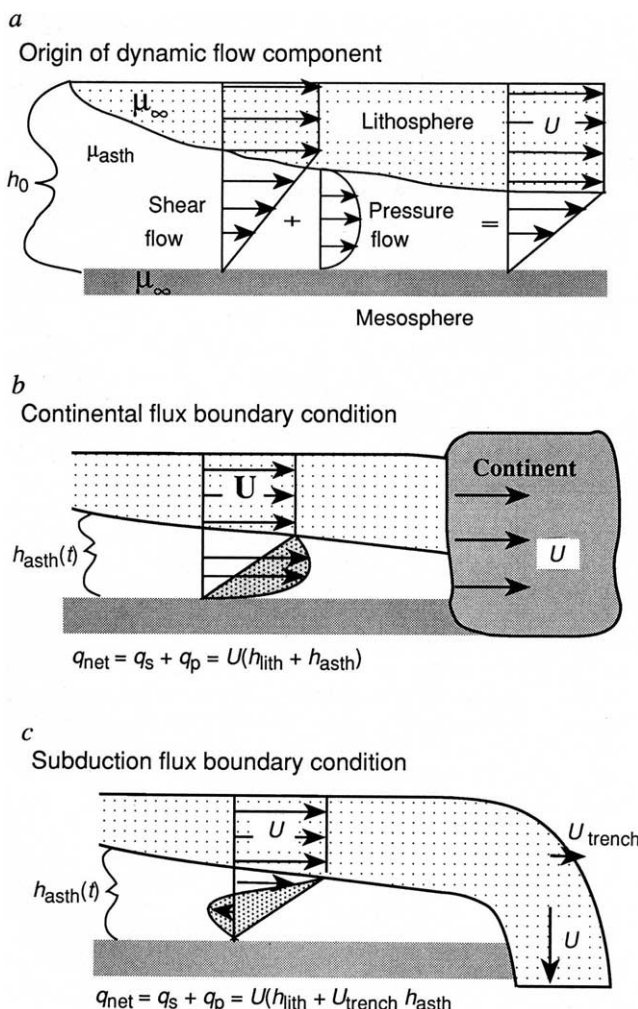


FIG. 1 a, Problem geometry for an asthenosphere-lithosphere system. Asthenosphere is bounded above by an isotherm (a temperature-dependent asthenosphere-lithosphere viscosity increase) and below by an isobar (a pressure-dependent asthenosphere-mesosphere viscosity increase). Plate cooling and thickening with age acts as a sink for asthenosphere. In this two-dimensional problem, the net horizontal flux of lithosphere plus asthenosphere is constant. Thus the incorporation of asthenosphere into a cooling, accreting lithosphere produces a dynamic pressure gradient which drives a pressure-induced flow component to replenish this lithosphere sink. b, Outflow boundary condition on asthenosphere flow for an oceanic subduction zone where only the lithosphere subducts. Also shown is an additional flow component if the trench is also migrating with respect to the mesosphere. c, Outflow boundary condition on asthenosphere flow for an ocean-continent margin where the net asthenosphere plus lithosphere flux is equal to the absolute plate motion times the total thickness of continental lithosphere and asthenosphere, $U_a h_{\text{lith}} + h_{\text{asth}}$. These differing outflow boundary conditions can lead to markedly different pressure-supported topography (Fig. 2a and b).

T_0 depends linearly on the magnitude of the absolute plate motion over the mesosphere and also on the rate of plate thickening with x which depends on the plate age, that is on U_r . This solution strongly depends on the net horizontal flux of material. A relatively thin asthenosphere channel could have very different flow if it were bounded by a continental margin instead of an oceanic subduction zone. At a 'thick-rooted' cratonic margin such as Australia, if the continental lithosphere (or 'tectosphere'²¹) extends to 'mesosphere' depths, the net asthenospheric flux $Q_{\text{tot}} = U_a h_0$ (Fig. 1b). In contrast, at a subduction zone if no asthenosphere is subducted, $Q_{\text{tot}} = U_a h_0(1 - \alpha(x_0))$ (Fig. 1c). In addition, the trench axis may migrate with respect to the mesosphere (Fig. 1c). To illustrate the effects of these different boundary conditions we consider two cases: (1) a Pacific subduction model which predicts flattening of the $t^{1/2}$ depth-age curve for older Pacific sea floor; and (2) a South Atlantic asymmetric subsidence model which predicts that the South American plate should subside more rapidly with age than the African plate.

In the subduction model we assume that the 'Pacific plate' has $U_a = U_r = 100 \text{ mm yr}^{-1}$, $h_0 = 200 \text{ km}$, $\mu = 2 \times 10^{18} \text{ Pa s}$ and that only lithosphere subducts at an age of 150 Myr, in other words that asthenosphere entrainment into the subducting slab is relatively small. This assumption is partially justified because the asthenosphere is less viscous than the mesosphere; it can be further justified if the asthenosphere is also less dense than the underlying mantle. Such a density contrast might be produced by a hotspot-fed asthenosphere that is $\sim 100^\circ \text{C}$ warmer than the mesosphere, and/or an asthenosphere that is compositionally less dense because it has experienced more melt extraction²¹. This parameter choice leads to the dynamic asthenosphere pressure effect shown by the dotted curve in Fig. 2a, which when added to a thermal halfspace subsidence of $300 \text{ m Myr}^{1/2}$ and an initial ridge elevation of $2,544 \text{ m}$ generates the predicted total subsidence shown by the solid line in Fig. 2a. This model predicts sea-floor flattening comparable to the deviations from $t^{1/2}$ cooling that are observed¹. It also predicts that the sea floor is simply compensated beneath young oceanic lithosphere but may be out of simple isostatic equilibrium beneath older sea floor, a signal which could show up as a discrepancy between the geoid over young and old Pacific sea floor. There is a geoid 'high' broadly associated with subduction zones which has previously been attributed to the regional presence of a cold strong slab²².

Sea floor created at a mid-ocean ridge often has asymmetric subsidence on one flank of the ridge compared with the other flank, although the flanks presumably share a common thermal origin at the spreading axis. In the South Atlantic, Hayes²³ has shown that the South American plate is subsiding more rapidly with age than the conjugate African plate side of the Mid-Atlantic Ridge. Figure 2b shows a swath of South Atlantic sea floor that originates from a single ridge and does not cover any of the main hotspot trails in this ocean basin. This region clearly shows the effect noted by Hayes²³, with the South American plate both moving and subsiding faster over the mesosphere than the African plate which is more nearly stationary in the hotspot reference frame. Figure 2b also shows the predicted thermal topography and asymmetric dynamic subsidence calculations for a uniform opening rate during the past 120 Myr, a South American migration rate $U_{\text{SA}} = 30 \text{ mm yr}^{-1}$ and $U_r = 20 \text{ mm yr}^{-1}$, an African migration rate $U_{\text{AF}} = 10 \text{ mm yr}^{-1}$ and $U_r = 20 \text{ mm yr}^{-1}$, an asthenosphere viscosity of $7 \times 10^{18} \text{ Pa s}$ and a maximum channel height $h_0 = 200 \text{ km}$ as in the previous example.

Other observations of the sea-floor bathymetry (and geoid) are consistent with this form of asthenosphere flow. Another good bathymetric example can be seen in Fig. 2c by examining depth contours near the Southeast Indian Ridge where the Indo-Australian plate subsides faster with age than does the Antarctic plate, which in turn has a much smaller motion over

the hotspot reference frame than does the rapidly moving Indo-Australian plate. In addition, this figure clearly shows the along-axis increase in depth of the Southeast Indian Ridge away from the Kerguelen and Amsterdam hotspots which may be the source of the asthenosphere for this section of the ridge²⁴⁻²⁶. This is an additional two-dimensional constraint on this hypothesis which will need testing numerically²⁰. Because of its linear dependence on the absolute motion of the lithosphere, the dynamic topography magnitude is intrinsically largest for relatively fast-moving plates in the Indian and Pacific ocean basins. Because of its additional dependence on asthenosphere resistance it will also be largest beneath old lithosphere with the thinnest (most resistive) asthenosphere. Thus the continental 'wake' effect caused by asthenosphere flow is evident (in the geoid) along the eastern margins of North and South America^{6,27} but is largest due south of the rapidly moving Indian Continent⁶. Topography and geoid highs are associated with hotspots¹⁷, consistent with the interpretation that hotspots are a source of asthenosphere.

The models presented above assume a uniform-viscosity asthenosphere whose thickness changes as a function of the age of the overlying cooling and thickening lithosphere. Although this is a defensible approximation which allows a simple closed-form solution, it is reasonable to question how well this treatment can approximate the net asthenospheric flow in a mantle whose viscosity depends on temperature and pressure. To assess this we assume a representative mantle viscosity structure²⁸ of the form $\mu = A e^{(E+PV)/RT}$, with an activation energy $E = 420 \text{ kJ mol}^{-1}$, activation volume $V = 10^{-5} \text{ m}^3 \text{ mol}^{-1}$. P and T are pressure and temperature, R is the ideal gas constant, and A is chosen so that $\mu = 10^{18} \text{ Pa s}$ at 150 km directly beneath the ridge axis. Slightly different temperature and pressure activation parameters will change the magnitude but not the overall geometry of the resulting viscosity-age dependence. With this more realistic viscosity structure it is possible to solve numerically the lubrication-theory equations for the dynamic flow to balance plate accretion. The net pressure flux is similar to that of the simpler parameterization even though our conception of what is the 'asthenosphere' in this flow regime must be reshaped. Instead of flow being confined to a constant-viscosity channel that thins with the growth of oceanic lithosphere, shear- and pressure-driven 'asthenosphere' flow is displaced to a deeper, higher-viscosity channel beneath older lithosphere. The growth of the lithosphere continually increases the depth and hence magnitude of the lowest-viscosity region in this system, thus increasing the effective resistance to asthenospheric flow with increasing age. We find that this net resistance is extremely similar in functional form to the $(h_0 - a\sqrt{x})^{-3}$ dependence in the analytic P - T treatment. Both decrease with the age of the lithosphere (they differ by a maximal factor of 2 for flow directly beneath a ridge axis). In the analytical solution this decrease with age occurs because the channel narrows while the viscosity is constant, whereas in the numerical solution the 'channel' remains nearly constant in thickness, but because it is displaced to greater depths beneath older, thick lithosphere, the average channel viscosity increases with age.

This treatment of the effects of asthenosphere flow on ocean depth-age and geoid-age relationships can be extended through a numerical formulation to include the two-dimensional effects of point-like hotspot sources of asthenosphere²⁰. One can investigate the importance of hotspot sources relative to broader-scale coupling of asthenosphere flow and mesosphere upwelling or downwelling at the asthenosphere-mesosphere boundary by incorporating this type of asthenosphere formulation into a three-dimensional model for lower-mantle flow^{29,30}. A critical question in the incorporation of asthenosphere flow into a broader convection model will be whether the asthenosphere is 'intrinsically' less dense due to thermal or compositional buoyancy than underlying mesosphere. This will affect the amount of asthenosphere entrainment into downwelling at an

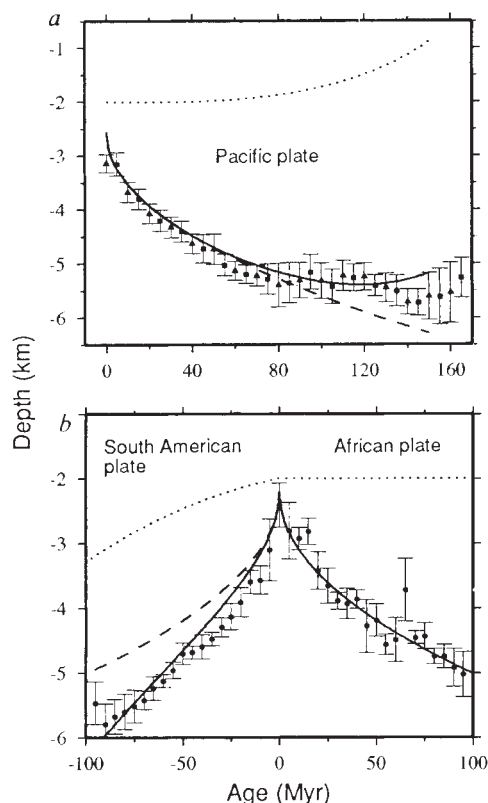
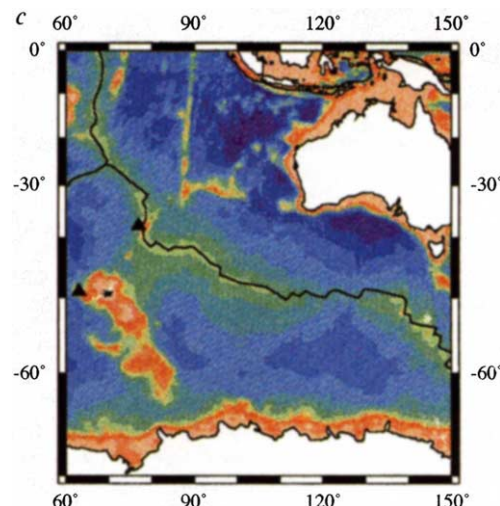


FIG. 2 *a*, Symbols show the modal (most frequent) depth of Pacific sea floor plotted as a function of age. Bars delimit the median absolute deviation from these modal depths. Squares show data based directly on shiptrack coverage from Smith¹¹ and triangles gridded data from ETOPO5³¹; for this study the two data sets do not differ significantly. The dotted line at the top shows the dynamic pressure contribution to topography as a function of age for the Pacific subduction model discussed in the text. A halfspace model of thermal boundary layer cooling $w(t) = -2.644 \text{ km} - 0.3 \text{ km (Myr)}^{-1/2} t^{1/2}$ is shown by the dashed line and the predicted net sea-floor relief shown by the solid line roughly matches the observed depth-age variation despite considerable differences between the true Pacific geometry and the two-dimensional plate boundary idealization inherent in the solution presented below. *b*, Bathymetry and best fitting depth as a function of age of Southern Atlantic Ocean sea floor for a tectonic corridor bounded by a northernmost flowline abutting the Gough fracture zone, a southern flowline abutting the Falklands escarpment, and isochrons



at 100 Myr on the African and South Atlantic plates. Depths are from NGDC ship data¹¹ and magnetic ages from Shaw and Cande³². Symbols same as above with the dotted line showing the dynamic pressure topography contribution as a function of age for the South American-African asymmetric subsidence model discussed in the text. A component of dynamic subsidence of the faster-moving South American Plate is able to explain the observed asymmetric differences in the subsidence of these two plates with age. *c*, The Southeast Indian ridge is a particularly simple hotspot and plate boundary configuration, illustrating several features that we suggest are a consequence of asthenosphere flow from the Kerguelen and Amsterdam hotspots (shown as triangles) to feed lithosphere growth to the east. The deepening of the ridge axis away from the Amsterdam hotspot is predicted by this flow scenario, as is the more rapid subsidence of the rapidly moving Indian plate with respect to the Antarctic Plate which is almost stationary in the hotspot reference frame (compare the distance of bathymetric contours north and south of the spreading axis).

oceanic subduction zone.

Although these investigations are desirable, they will all involve complex numerical studies. The simple solution presented here allows us to explore the effects of asthenosphere flow and gain insight into their patterns and magnitude beneath

moving, accreting oceanic lithosphere. The success of this treatment in generating sea-floor bathymetry anomalies such as the observed sea-floor flattening with age and depth-age asymmetries about mid-ocean ridges suggests that a more complete investigation of these effects is warranted. □

Received 26 March; accepted 11 August 1992.

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ACKNOWLEDGEMENTS. J. Morgan provided helpful advice about this work. This research was partially supported by the NSF. W.H.F.S. gratefully acknowledges the support of the Cecil and Ida Green Foundation.

Marsupial Y chromosome encodes a homologue of the mouse Y-linked candidate spermatogenesis gene *Ube1y*

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THE mammalian subclass Theria consists of infraclass Meta-theria (marsupials) and Eutheria ('placentals') which diverged from each other 120–150 million years before present¹ (Myr BP). Both infraclass have Y chromosome-dependent testis determination but direct molecular evidence linking the Metatherian and Eutherian Y chromosomes is lacking. Comparative analyses indicate that three mammalian genes have remained Y-linked for at least 80 Myr, since the divergence of the Eutherian orders from a common ancestor. These are *Zfy*, a gene encoding a transcription factor of the zinc-finger type²; *Sry*, the putative primary testis-determining gene³; and *Ube1y* (formerly *Shy* or *Alis9Y-1*), a candidate for the mouse spermatogenesis gene *Spy*, encoding a ubiquitin-activating enzyme E1 homologue^{4,5}. Although in marsupials *Zfy* homologues are autosomal⁶, a Y homologue of *Sry* has recently been isolated⁷. We report here the identification of a functional marsupial Y-linked homologue of the murine *Ube1y* gene establishing that Metatherian and Eutherian Y chromosomes diverged from a common ancestor. This extreme conservation indicates that *Ube1y* plays a critical role in male development.

Ube1y-related sequences were initially detected in marsupials by hybridizing a portion of a complementary DNA for the homologous mouse X-linked *Ube1x* to a Southern blot of male and female genomic DNA from three marsupial species; the macropodid family members *Macropus rufus* (red kangaroo)

FIG. 1 Southern blot analysis of marsupial DNA with the mouse *Ube1x* cDNA, pCR5 (ref. 4). *a*, From left to right, *Eco*RI digests of male and female DNA from *M. rufus*, *M. eugenii* and *S. macroura*. Clear male-specific bands (arrowed) can be seen in all three species, at 2.5 kb and 1.4 kb (*M. rufus*), 2.4 kb and 1.5 kb (*M. eugenii*), and 6.4 kb and 1.0 kb (*S. macroura*) as well as male–female common bands. Some of these latter fragments (starred) show male–female dosage differences consistent with X-linkage. *b*, Marsupial–rodent somatic cell hybrid panels from the macropodid *M. rufus* (left) and the dasyurid *P. maculata* (right) digested with *Eco*RI and hybridized with pCR5. The tracks are: 1, marsupial parent line; 2, marsupial X-containing hybrid (+); 3, X hybrid revertant lacking marsupial X (–); 4, female mouse (*M. rufus*) or female Chinese hamster (*P. maculata*). In *M. rufus* there is a 2.0-kb band present in the X hybrid but absent from the revertant which must map to the X chromosome. In *P. maculata* there is a 10-kb band shared with the parent line and the X hybrid that is missing from the revertant. This band defines an X homology. There is also however, a 5.2-kb band in the parent line that is not in the X hybrid. This band defines an autosomal homology. METHODS. *M. rufus* male and female DNA was hybridized at 60 °C and washed in 2 × SSC, 55 °C as previously described⁴. The other blots were made on Hybond N+ (Amersham). These were hybridized in 6 × SSPE, 10% dextran sulphate at 65 °C and washed in 2 × SSC, 65 °C. The pCR5 probes were labelled with ³²P by random priming¹⁸. All blots were hybridized with the full 1.6-kb insert of pCR5 except the *M. rufus*

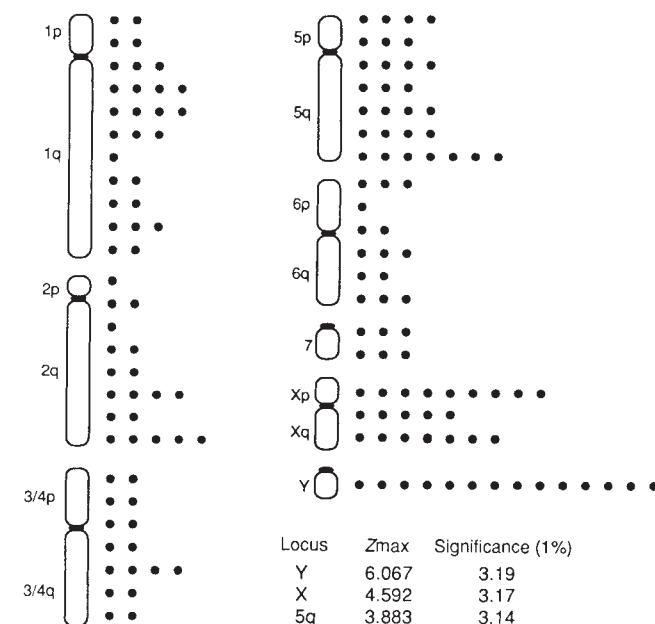
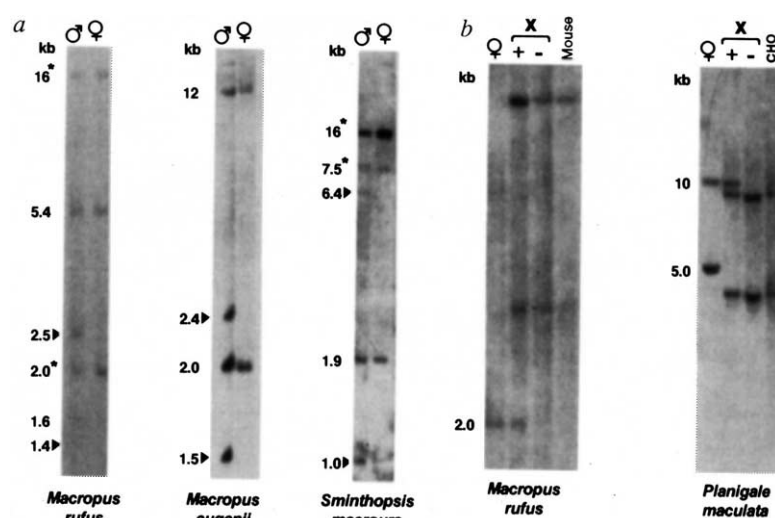


FIG. 2 Mapping *Ube1y* homologues in a *Macropus eugenii* male by *in situ* hybridization. Using the Zmax statistical analysis¹⁹ three significant (1%) points of localization were identified. In order of significance, these were on the Y, X and 5qter. When scored separately both Xp and Xq have significant points of localization but, given the small size of the X (3% of the haploid genome), two sites cannot be resolved on the data.

METHODS. The ³H-labelled *Ube1x* cDNA clone pCR5 in pBluescript II (Stratagene) was hybridized at 0.1 µg ml⁻¹ to metaphase chromosomes as previously described²⁰. One-hundred spreads were observed and 267 grains scored.

and *Macropus eugenii* (tammar wallaby), and a member of the dasyurid family *Sminthopsis macroura* (striped-face dunnart). Male-specific fragments were seen in all three cases (arrowed in Fig. 1a). In addition, several male–female common fragments could be detected, dosage of which was consistent with X linkage and autosomal linkage. Assignment of the X chromosome was

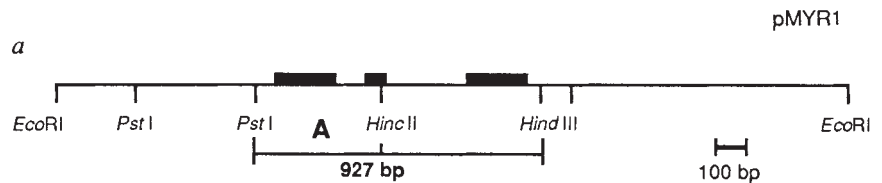


FIG. 3 Sequence analysis of the *M. rufus* male-specific 2.5-kb *EcoRI* fragment, pMYR1. **a**, Schematic of pMYR1 showing the position of the 927-bp *PstI*-*HindIII* fragment sequenced. The location of the 411-bp *PstI*-*HincII* fragment, pMYR1.A, is marked with an 'A'. The positions of regions with homology to *Ube1y* are represented by the boxes. Further homologous sequences, not characterized here, lie to the right of the 927-bp *PstI*-*HincII* fragment. **b**, Sequence of the 927-bp *PstI*-*HindIII* fragment of pMYR1. Three putative exons with homology to *Ube1y* are shaded and consensus splice sites bordering these exons are boxed. The amino acids encoded by these exons are indicated. **c**, Comparison of the deduced peptide encoded by *Ube1y* (*M.m.musculus*) and pMYR1 (*M. rufus*). The *M. rufus* peptide was derived after splicing the three conserved regions of pMYR1. Only one frame maintained on ORF and encoded the 152-amino-acid peptide shown. Lines indicate identity and dots conservative substitutions.

METHODS. The 2.5-kb *M. rufus* male-specific fragment was isolated from a λ gt10 library of size-fractionated male DNA. *EcoRI*-digested *M. rufus* DNA was run overnight on a 0.8% agarose gel, the fragments of ~2-3 kb were excised and the DNA isolated as previously described²¹. The isolated fragments were cloned into the *EcoRI* site of λ gt10. The library was screened with pCR5.A, C and D. Several positive clones contained inserts of 2.5 kb, one of which was subcloned into *EcoRI*-cut pBluescript II (Stratagene) and termed pMYR1. Sequencing was done as previously described⁴. Plasmids with MYR1 in both orientations were partially deleted by digestion with *HincII*, *HindIII* or *PstI* followed by recircularization. They were then sequenced using T3 and T7 primers. Sequence comparisons were done using the University of Wisconsin GCG sequence analysis software²².

b

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1 CTGCAGTATCTGTCAATCACACATCTGCTTTTCTTAAGGGTTCCTTTCTTCTTTTACTT
  --MetTyrMetAspArgHisCysValTyrTyrArgLysProLeuLeuGluSerGlyT
61 CAGGAATGTACATGGACAGACATTGTGTGTACTATCGGAAACCACTGCTAGAGTCAGGCA
  hrLeuGlyThrLysGlyAsnIleGlnValValIleProPheLeuThrGluSerTyrSerS
121 CACTGGGAACCAAGGGAAATATTACAGGTGGTGATACCCCTTCTGACAGAATCATACAGCT
  erSerGlnAspProProGluLysSerIleProIleCysThrLeuLysAsnPheProAsnA
181 CCAGCCAGGACCCCTGAGAAATCCATCCCATCTGTACTACTCAAGAACCTTCCCAATG
  laIleGluHisThrLeuGln
241 CCATCGAGCACACACTTCAGGTCTGGGAAATGGATCAGGGACTTAGGGAGGTTTTATTG
301 GGGAGGTGGGAGTAATTAGGTCACTTCCTCTTAATAAAATCCATATTCTGCTGCACGCA
  TrpAlaArgAspGluPheGluSerLeuPheLysGlnProAlaGluAsnValAsnGlnTy
361 GTGGGCCGAGATGAGTTTGAAGCCTCTCAAACAACCAGCAGAAAATGTAAACCAATA
  rLeuTh
421 CCTGCTGAGTAGTTGCTGGTTCCTTACCCATAATGCTAGATCCCTGGCCTAGGCTAC
481 ACTAGACCAGGCATCTTTTGCTGTTTTCTATTCTCTATGGACTTAATTTACAACCCCA
541 TTTGAGACTCCATGTGTCCCATCTCTAAGATATTACATTCTGTTTATTGTTTGTACTT
601 AATGCTGTAGATTGTGCAGATTTTATCTTCAGACTTTTGAGTGACTGTTTTCCAACTA
  rAsnProLysPheValGluArgThrLeuAr
661 ACTATCAACTTTTGTCTATTATCTTACAGGAACCCAAATTTGTGGAGAGAACTCTGAG
  gLeuGlyGlyThrGlnProLeuGluValLeuGluAlaValHisArgSerLeuValLeuGl
721 ACTGGGAGGGACACAACCCCTGGAGGTGCTAGAAGCTGTACATCGTAGCTTGGTGTGCA
  nArgProHisAspTrpAlaAspCysValThrTrpAlaCysLeuHisTrpHisSerGlnTy
781 GCGCCCCATGACTGGGCTGATGTGTGACTTGGGCTGCCTTCATTGGCATTCTCAGTA
  rAlaAsnAsnIleArgGlnLeuLeuHisAsnPheProProGluGln
841 TGCCAACAATATCCGTCAGCTCTGCATAACTTTCTCCAGAACAGGTAATCAACTAATT
901 CTTTATCTTTTCAAGTTCATTTAAGCTT
  
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c

<i>M. rufus</i>	MYMDRHCVVYRKPLLESGLTGTGKNIQVVFPLTESYSSSQDPPEKSIP I
<i>M.m.musculus</i> Y	LYVDRRCVVYRKPLLESGLTGTGKNVQVVFPLTESYSSSQDPPEKSIP I
<i>M. rufus</i>	CTLKNFPNAIEHTLQWARDEFESLFKQPAENVNQLTNPKFVERTLRLGG
<i>M.m.musculus</i> Y	CTLKYFPNAIEHTVQWARDEFEGFLFKQSAENVNQLTDPKFMERTLQLAG
<i>M. rufus</i>	TQPLEVLEAVHRSVLQRPDWDACVTVWACLHWHSQYANNIRQLLHNFPF
<i>M.m.musculus</i> Y	TQPLEVLEAIHCSVLQRPQTWADCVTWAYQHWHTQYSHNIQQLLHNFPF
<i>M. rufus</i>	EQ
<i>M.m.musculus</i> Y	AQ

formally demonstrated for the macropodid *M. rufus* (2.0 kilobases (kb)) and the dasyurid *Planigale maculata* (10 kb) (common planigale) using somatic cell hybrids which retained a marsupial X chromosome (Fig. 1b). The hybrids also revealed a clear autosomal homology in *P. maculata* (5.2 kb) but not in *M. rufus*.

Y-linkage of the male-specific bands detected by Southern analysis in *M. eugenii* was confirmed by *in situ* hybridization of a *Ubelx* cDNA to metaphase chromosomes. Major hybridization sites were found on the Y and X with a minor site on 5qter (Fig. 2). Taken together these data show that macropodidae and dasyuridae possess sequences related to *Ubelx/Ubel1* on the Y and X chromosomes and an autosome. Because these marsupial families diverged some 50 Myr BP¹, it is likely that they have remained conserved on the Y and X in all marsupials. The significance of the autosomal linkage is unclear.

To assess whether the marsupial Y locus encodes a functional gene, the male-specific 2.5 kb *EcoRI* fragment (Fig. 3a) was cloned from *M. rufus*. Regions conserved with *Ubelx* were mapped into a 927-base-pair (bp) internal *PstI*-*HindIII* fragment which was then sequenced (Fig. 3b). This revealed three regions that were 77% identical with the mouse Y cDNA adjacent to regions completely lacking in identity. These were concluded to be exons and introns, respectively, and their positions are indicated in Fig. 3a and b. Consensus splice sites were identified at the ends of these conserved regions (boxed in Fig. 3b) and were used to theoretically splice the 'exons'. This 'spliced messenger RNA' was then translated, revealing a single open reading frame (ORF) encoding a 152-amino-acid peptide which is 84% identical and 93% similar to the mouse *Ubel1* (Fig. 3c). It is slightly more conserved with eutherian X homologues, having ~90% identity and ~96% similarity to the mouse *Ubelx* and the human *UBE1*. To rule out the possibility that this gene is the result of a recent transposition from the X or an autosome, the 411-bp *PstI*-*HincII* fragment pMRY1.A (Fig. 3a), containing a 195-bp exon and flanking intronic sequences, was hybridized at low stringency to male and female *M. rufus* DNA (Fig. 4). Under these conditions the probe is totally Y-specific. This gene has thus maintained an ORF and splice sites while diverging from the X and autosomal homologues. With the presence of *Ubel1*-related sequences on the Y chromosome in other marsupial species, this strongly

argues that there is a functional ubiquitin-activating enzyme homologue on the marsupial Y chromosome.

Although the mouse *Ubel1* gene identifies Y homologues in marsupials and all non-primate Eutherians tested, no human or chimpanzee Y sequences can be detected⁴. It therefore seems likely that the Y gene has been lost in higher primates. The X chromosome allele in these primates may have evolved to compensate for this lack of a *Ubel1* Y gene, the expression of which would still be required in most other species. Examples of differences between mouse and human transcriptional control exist in three X genes, which have been shown to escape X inactivation in the human but not in the mouse (*Rps4X*^{8,9}, *Ubelx*^{5,10} and *Zfx*^{9,11}). Interestingly, the human Y-linked ribosomal gene *RPS4Y*⁸ presents the converse situation to *Ubel1*, having no detectable homologue on the mouse Y chromosome^{9,12}. These inconsistencies may be a consequence of the same processes, now acting independently on X-Y common genes in the different Eutherian lineages, that resulted in the removal of much of the coding capacity of the Y chromosome as the X and Y evolved from an autosomal pair present in a common ancestor of Metatherians and Eutherians.

Comparative mapping of Eutherian X and Y genes in Metatherians has revealed that genes from human Xq have X-linked homologues in marsupials¹³ and monotremes²³, but genes mapping to the human Xp are autosomal in these species^{14,15}. It has been suggested that this latter region has been acquired from the autosomes in the 40-70 Myr separating the Metatherian and Eutherian progenitors and that human Xq represents an ancestral form of the Therian X chromosome¹⁵. If the X and Y chromosomes have gained autosomal sequences during evolution, it can be concluded that *Zfy* and *Zfx*, which are autosomal in marsupials⁶, must be a more recent addition to the sex chromosomes than *Ubel1* and *Ubelx*, which map to the marsupial sex chromosomes. The finding of *Ubel1* homologues on the marsupial X suggests that the ancestral X also included sequences located at human centromeric Xp and predicts that *Ubel1* homologues should map to Xp in marsupials. *Ubel1*-related X genes are closely linked to *Syn1* in the mouse and the human^{4,5,16}. By contrast, in marsupials *Syn1* homologues are autosomal¹⁴. These data pinpoint an evolutionary breakpoint between *Syn1* and *Ubelx* on the Eutherian X chromosome, which will be refined by mapping other markers with homologues on human Xp. The mapping of a *Ubel1* homologue to the marsupial Y chromosome establishes, at the molecular level, that the Eutherian and Metatherian Y chromosomes have a common origin. Therefore if 'Sry' is the Eutherian primary testis-determining gene, it should also be located on the testis-determining marsupial Y chromosome. The recent identification of a marsupial Y-linked *Sry* homologue confirms this prediction⁷. These data show that the *Ubel1* and *Sry* genes have been conserved on the mammalian Y chromosome for at least 120-150 Myr. Such extreme conservation of *Ubel1* indicates that, with the apparent exceptions of human and chimpanzee, *Ubel1* Y homologues play important roles in male development in a wide variety of therian mammals. In the mouse it has been hypothesized^{4,5} that this role is the same as that of the Y-linked spermatogenesis factor *SpY*, necessary for the completion of the initial mitotic stages of spermatogenesis¹⁷. □

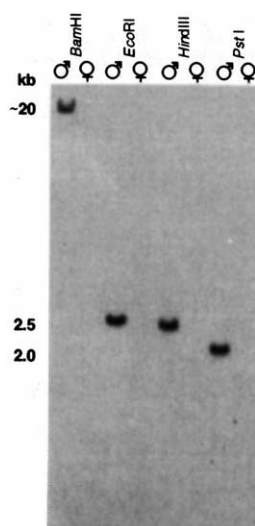


FIG. 4 Southern hybridization analysis of *M. rufus* male and female genomic DNA with pMRY1. A. The genomic DNA was cut with *Bam*HI, *Hind*III and *Pst*I. Hybridization is only seen to the male DNA.

METHODS. Southern analysis as in Fig. 1 legend. Post-hybridization washes were in 2 × SSC at 55 °C.

Received 23 March; accepted 14 August 1992.

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ACKNOWLEDGEMENTS. We thank M. Douglass of Memphis Zoo for *M. rufus* blood samples, J. L. Simpson, S. Elias and the UT Medical Group for their support and R. Dodd of UTMG Graphics. This work was supported by the NIH (C.E.B.).

Evolution of sex determination and the Y chromosome: *SRY*-related sequences in marsupials

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IN mammals, testis determination is under the control of the testis-determining factor borne by the Y chromosome^{1,2}. *SRY*, a gene cloned from the sex-determining region of the human Y chromosome, has been equated with the testis-determining factor in man^{3–5} and mouse^{6,7}. We have used a human *SRY* probe to identify and clone related genes from the Y chromosome of two marsupial species. Comparisons of eutherian and metatherian Y-located *SRY* sequences suggest rapid evolution of these genes, especially outside the region encoding the DNA-binding HMG box. The *SRY* homologues, together with the mouse *Ube1y* homologues⁸, are the first genes to be identified on the marsupial Y chromosome.

Marsupials (mammalian Infraclass Metatheria) diverged from eutherian ('placental') mammals 80–180 Myr before present⁹. In marsupials, as in eutherian mammals, the Y chromosome is testis-determining, although scrotum development and some other sexual dimorphisms may be controlled by X chromosome dosage^{10,11}. A gene regulating testis determination and derived from a common mammalian ancestor would be expected to be conserved in sequence between eutherians and marsupials and to map to the marsupial Y chromosome^{12,13}.

Sminthopsis macroura (Order Polyprotodonta, Family Dasyuridae), and *Macropus eugenii* (Order Diprotodonta,

Family Macropodidae) are representatives of two major Australian marsupial Orders, which diverged from each other 40–80 Myr ago. A human *SRY* probe, a 0.9-kilobase (kb) *HincII* subclone of pY53.3 (ref. 3), was used to identify homologues of this gene by Southern hybridization in these two species. The probe detects male-specific restriction fragments in both species as well as several fragments shared by males and females (Fig. 1a). The male-specific fragments were presumed to represent marsupial *SRY*-homologous genes borne on the Y chromosomes, and the shared fragments, autosomal *SRY*-like sequences (*SOX*). These genes have been described previously in mouse⁶, rat¹⁴, man¹⁵, *Drosophila*¹⁵ and birds¹⁶.

The human *SRY* probe was used to screen a complementary DNA library constructed from *S. macroura* adult testis RNA. One positive clone, pcSmY1.4, was isolated and used to probe genomic Southern blots. At high stringency it hybridizes to DNA

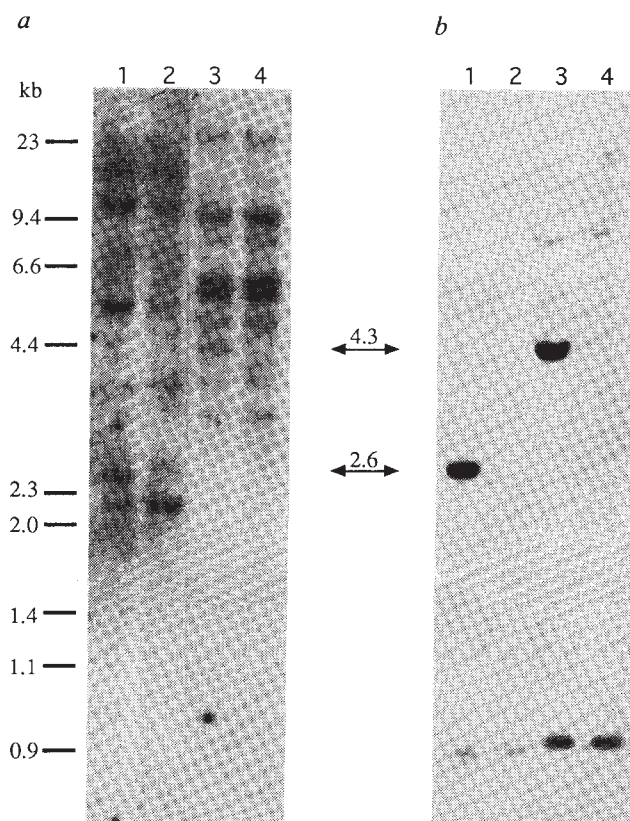


FIG. 1 Identification of male-specific and male/female shared marsupial sequences related to the human *SRY* gene. Southern blots of *EcoRI*-digested genomic DNA from male and female pairs of *S. macroura* and *M. eugenii* were hybridized with human and *S. macroura* Y-linked *SRY* homologues. Lane 1, *S. macroura* male; lane 2, *S. macroura* female; lane 3, *M. eugenii* male; lane 4, *M. eugenii* female. Sizes are given in kb. Male-specific bands at 2.6 and 4.3 kb are arrowed. a, A 0.9-kb *HincII* fragment of pY53.3 containing the human *SRY* ORF detects several bands shared between males and females of each species, as well as male-specific bands of 2.6 kb (in *S. macroura*) and 4.3 kb (in *M. eugenii*). b, A 0.6-kb *EcoRI* fragment derived from pcSmY1.4, containing sequences homologous to pY53.3, detects a 2.6-kb fragment in *S. macroura* male DNA, and a 4.3-kb fragment in *M. eugenii*. This probe also detects additional fragments not homologous to the human probe.

METHODS. For Southern blots genomic DNA was digested with *EcoRI*, electrophoresed through a 0.8% agarose gel, transferred with 20×SSC to Hybond N+ (Amersham) and fixed in 0.4% NaOH. Probes were labelled with [³²P]dCTP and added to the filter in 5×SSPE (1×SSPE: 0.15 M NaCl, 0.01 M NaH₂PO₄·H₂O, 0.1 mM EDTA, pH 7.4), 5×Denhardt's solution, 0.5% SDS, 200 μg ml⁻¹ denatured salmon sperm DNA (10% dextran sulphate was used in the case of the human probe) and hybridized for 20 h at 65 °C. Filters were washed in 2×SSC, 0.2% SDS at 65 °C and autoradiographed for 10–40 days (pY53.3) or 1–3 days (pcSmY1.4).

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NATURE • VOL 359 • 8 OCTOBER 1992

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ACKNOWLEDGEMENTS. *S. macroura* and *M. eugenii* material was obtained under the provisions of the Victoria Department of Conservation and Environment, permit no RP-90-177. We thank E. Tanger for maintenance of the *S. macroura* colony and G. Shaw for assistance with *M. eugenii* material. Supported by grants from the National Health and Medical Research Council and the Australian Research Council (J.M.G.), the UK Medical Research Council (R.L.-B.) and the Imperial Cancer Research Fund (P.N.G.). G.K.H. was the recipient of a US NSF Postdoctoral Fellowship and J.W.F. of an Australian Postgraduate Research Award.

Microtubule-motor activity of a yeast centromere-binding protein complex

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DURING cell division, sister chromosomes segregate from each other on a microtubule-based structure called the mitotic spindle. Proteins bind to the centromere, a region of chromosomal DNA, to form the kinetochore, which mediates chromosome attachment to the mitotic spindle microtubules^{1,2}. In the budding yeast *Saccharomyces cerevisiae*, genetic analysis has shown that the 28-base-pair (bp) CDEIII region of the 125-bp centromere DNA sequence (CEN sequence) is the main region controlling chromosome segregation *in vivo*^{3,4}. Therefore it is likely that proteins binding to the CDEIII region link the centromeres to the microtubules during mitosis. A complex of proteins (CBF3) that binds specifically to the CDEIII DNA sequence has been isolated by affinity chromatography⁵. Here we describe kinetochore function *in vitro*. The CBF3 complex can link DNA to microtubules, and the complex contains a minus-end-directed microtubule-based motor. We

suggest that microtubule-based motors form the fundamental link between microtubules and chromosomes at mitosis.

A single point mutation in the CDEIII region of the centromere, RN2011, dramatically decreases the fidelity of chromosome transmission^{4,6}. The CBF3 complex of proteins binds to a DNA-affinity column containing CEN DNA sequences, but not an affinity column containing CEN DNA sequences with the RN2011 point mutation, indicating that the CBF3 proteins may mediate chromosome segregation *in vivo*⁵. We isolated the CBF3 complex of proteins by affinity chromatography using a 350-bp DNA fragment containing the CEN DNA⁵. The proteins eluted from mutant and wild-type CEN DNA affinity columns are shown in Fig. 1a. Three major proteins (*M*, 110K, 64K and 58K) and some minor proteins elute from the wild-type but not the mutant DNA column, and these are therefore defined as the CBF3 complex of CDEIII binding proteins⁵.

To see if the CBF3 proteins eluted from the wild-type CEN DNA column could bind to microtubules we used the assay illustrated in Fig. 1b. Biotinylated CEN DNA was attached to streptavidin-bearing fluorescent beads, and the resulting CEN DNA beads were incubated with the purified CBF3 proteins. A competition assay showed that the CBF3 proteins (WT in Fig. 1a) were binding to the CEN DNA beads. The DNA-binding activity of the CBF3 proteins was measured by analysing complexes formed between CBF3 proteins and radioactive CEN DNA on non-denaturing gels^{4,5}. Incubation with CBF3 retards the gel mobility in a characteristic manner (Fig. 1c, lane 1). Pre-incubation of the CEN DNA beads with CBF3 greatly reduced complex formation between CBF3 and labelled CEN

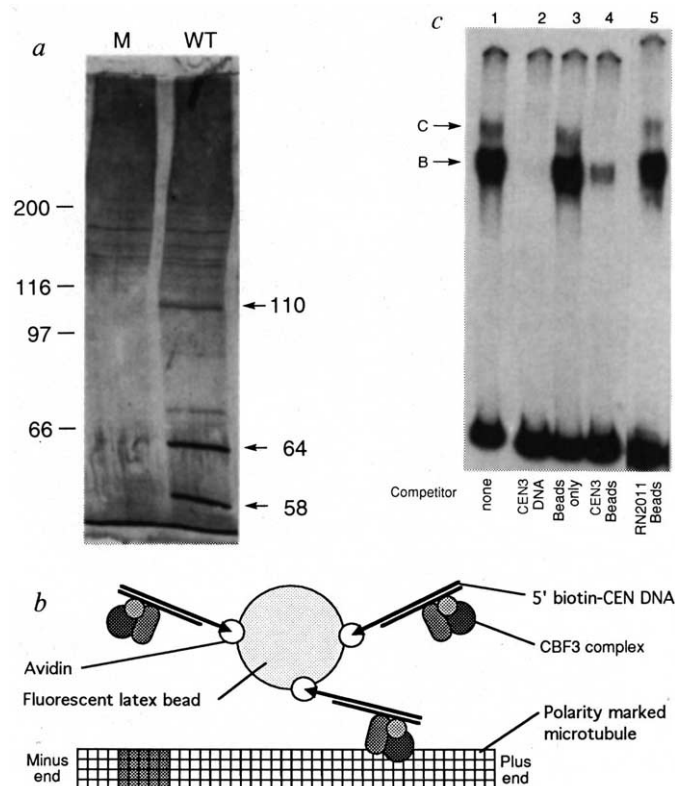
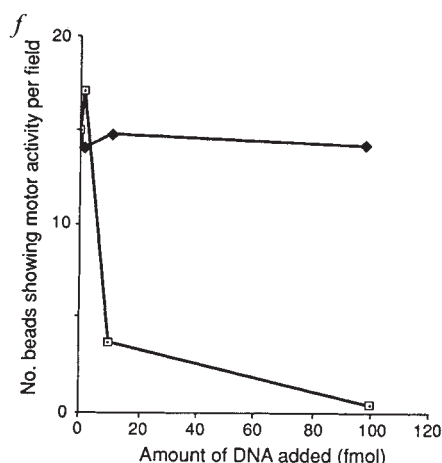
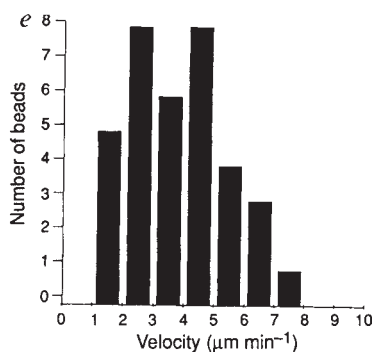
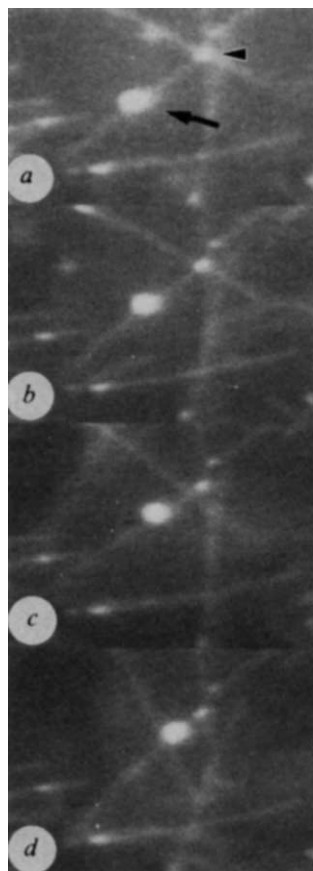


FIG. 1 a, Silver-stained gel showing the proteins eluting from a wild-type (WT) and an RN2011 (M) DNA affinity column. Bands appearing between the proteins migrating at *M*, 110K and 64K are likely to be 110K breakdown products and are not seen consistently in different preparations. CBF3 protein (20 μ l; 1/25 of the total preparation, representing 300 fmol of CEN DNA-shift activity in the wild-type preparation), was loaded for each lane of a 10% SDS-polyacrylamide gel and electrophoresed for 1 h at 180 V (ref. 5), and subsequently silver-stained as described¹⁹. CBF3 protein was prepared as described⁵, except that NaF was omitted from the final elution buffer of the affinity column. b, Diagram of the assay for the microtubule-binding activity of the CBF3 complex. c, A gel assay for the mobility shift of a DNA fragment, used to show that CEN DNA-coated rhodamine beads can bind CBF3 protein. Affinity-purified CBF3 proteins (1 μ l) were preincubated in binding buffer⁵ at room temperature for 10 min with the following competitors: lane 1, none; 2, 350-bp CEN3 DNA fragment (160 fmol); 3, 10⁶ beads only; 4, CEN3 beads (160 fmol 350-bp wild-type CEN3 DNA fragment on 10⁶ beads); 5, RN2011 beads (160 fmol of a 350-bp mutant RN2011 CEN3 fragment⁴ on 10⁶ beads). End-labelled (³²P) 350-bp CEN3 DNA fragment was added to each tube and after a further 10 min, the reactions were analysed on a 4% acrylamide gel as described⁵. Shifted bands B and C are characteristic of CEN3 DNA/CBF3 complexes⁵.

METHODS. 0.2- μ m carboxylated rhodamine beads (molecular probes) were derived using an EDAC (1-ethyl-3-(3-dimethyl aminopropyl)-carbodiimide) coupling with streptavidin. Binding of streptavidin to the beads was checked using [³H]biotin. The 350-bp *Xba*I-*Eco*RI DNA wild-type CEN fragment from pRN505 (ref. 4) or the RN2011 point mutation in which converts the central CCG in CDEIII to CCC (ref. 4) was biotinylated as described⁵. A proportion was also end-labelled with ³²P (ref. 5). Biotinylated DNA was added to the beads, and the beads were then centrifuged at 13,000g for 10 min. ³²P-biotinylated DNA was then added to check for saturation of the beads with DNA. The gel-shift assay was performed as described⁵ with the following buffer: 10 mM HEPES, pH 8.0, 10% glycerol, 6 mM MgCl₂, 100 mM KCl.



DNA molecules (lane 4), whereas beads without bound DNA did not compete in the assay (lane 3).

To assay microtubule-binding by CEN DNA beads with attached CBF3 proteins (CBF3 beads), rhodamine-labelled, taxol-stabilized microtubules were attached to the glass surface of a thin perfusion chamber⁷. CBF3 beads were introduced into a chamber containing attached microtubules and incubated for 5 min. Under these conditions, CBF3 beads bound to microtubules, whereas CEN DNA beads in the absence of protein did not bind. An example of a bead-microtubule complex is shown in Fig. 2a. The bead is marked with an arrow and can easily be distinguished from the smaller microtubule.

Microtubule-based motors have been implicated in the mechanisms of kinetochore-microtubule interaction in mammalian cells⁸⁻¹¹. To test whether CBF3 beads bound to microtubules using a microtubule motor, caged ATP, which cannot be hydrolysed by ATPases, was introduced to the preparation by perfusing the chamber. Irradiation with ultraviolet light releases free ATP from the caging groups¹². Individual bead-microtubule complexes were identified by rhodamine fluorescence, and briefly irradiated with light of 360-nm wavelength to activate the ATP. After uncaging, most of the beads moved along the microtubules at an average rate of $4.1 \pm 1.6 \mu\text{m min}^{-1}$ (Fig. 2e). Different motor proteins move towards either the plus- or minus-ends of microtubules (for example, kinesin moves

FIG. 2 CBF3 exhibits a microtubule-based motor activity. *a*, A bead (arrow-head) attached to a microtubule, the minus-end of which is marked by a bright rhodamine seed (arrow). *b*, After generation of ATP. The bead has moved along the microtubule towards the minus-end. *c*, *d*, Further movement of the bead. Four different preparations of CBF3 protein exhibited this microtubule motor activity. *e*, Histogram of velocities of CBF3 beads. *f*, Graph of the microtubule-motor activity of CBF3 beads as a function of the concentration of soluble CEN DNA added. □, Soluble wild-type CEN DNA; ♦, soluble RN2011 point-mutant CEN DNA. The assay was performed as in *a*, except that varying amounts of wild-type or RN2011 point-mutation DNA in 10 mM Tris, 1 mM EDTA, pH 8.0, was added with the CBF3. Bead activity was determined as in Table 1. CBF3 protein was purified on an affinity column using an 80-bp synthetic oligonucleotide encompassing the CDEIII region, 19-bp of CDEII and 36-bp of continuous CEN3-flanking DNA.

METHODS. Beads were prepared as in Fig. 1. The density of CBF3 protein on the beads was adjusted as >20 molecules of kinesin are required to see efficient movement of the beads along the microtubule²⁰. As a CBF3 protein preparation can bind 5–10 fmol DNA per μl (data not shown), we used 10^7 beads per reaction, and added $1 \mu\text{l}$ of protein, so that ~ 100 molecules CBF3 should be bound per bead⁵. Beads were added to the reaction buffer (10 mM HEPES, pH 8.0, 10% glycerol, 6 mM MgCl_2 , 0.1 mg ml^{-1} salmon sperm DNA, 5 mM dithiothreitol, 1 mg ml^{-1} casein) and left for 15 min for the casein to block the bead surface against protein binding. CBF3 protein was then added to 1/10 final volume and KCl (100 mM) to give the final volume. The mixture was left to incubate for 45 min, and then the mixture diluted 1:1 with 0.05 mg ml^{-1} glucose oxidase, 0.1 mg ml^{-1} catalase, 10 mM glucose, 0.1% β -mercaptoethanol, 1 mM MgCl_2 , 10% glycerol in 10 mM HEPES, pH 8.0 (antifade buffer), to a final KCl concentration of 50 mM. Perfusion chambers were prepared by placing two layers of grease containing 18- μm latex beads on a slide and a coverslip placed over (final chamber volume $\sim 1.5 \mu\text{l}$). Polarity-marked microtubules were prepared as described⁷. They were perfused into the chamber, where they stuck only by their labelled seed, and the chamber immediately washed with 5 mg ml^{-1} casein in 50 mM Tris, pH 8.0. After 5 min, the CBF3 CEN DNA-bead complex and antifade was perfused in and left for 5 min, and then washed out in the antifade buffer. The attached beads and microtubules were observed with a Zeiss standard microscope by epifluorescence. To count the number of attached beads, fields were selected at random. A bead was counted as bound if it was attached to a microtubule that was tethered only by its bright seed. To initiate bead movement, 1 mM NPE-caged ATP (Molecular Probes) in the antifade buffer was perfused in. A 0.1 neutral density filter was placed in the excitation pathway, and beads attached to microtubules were selected using a silicon intensified tube camera. Recording was started, using shuttering to minimize the light dose, then the filter was removed and the fluorescence channel switched to the Hoechst to release ATP. The filter was replaced and any subsequent movement recorded in the rhodamine channel. Details of the microscope system have been described elsewhere⁸.

TABLE 1 Microtubule binding and motor activity of CBF3 protein and DNA-coated beads

	Protein origin		Beads with attached RN2011	
	Wild-type CEN DNA affinity column	RN2011 CEN DNA affinity column	CEN DNA	CEN DNA
Microtubule-attached beads/fields	3.2	0.11	0.5	0.08
Beads with active microtubule-based motors	12/21	1/10	29/33	5/32
Active beads/field	1.8	0.011	0.44	0.0125

Microtubule-binding and motor activity of CBF3 protein purified on an RN2011 CEN DNA affinity column compared to that purified on a wild-type CEN DNA affinity column, and the activity of beads with an RN2011 point mutant CEN DNA sequence or a wild-type CEN DNA sequence attached. Protein was prepared as for Fig. 1, except that a single preparation was divided after the S200 sizing column⁵ and half each run over an affinity column containing a 330-bp wild-type CEN fragment, or a column with the 350-bp fragment containing the RN2011 point mutation. Gel-shift assays confirmed very little gel-shift activity from the protein eluted from the RN2011 column, as previously described⁵ (data not shown). For the microtubule-motor activity, the reaction was set up as in Fig. 2 and perfused into a chamber containing bound microtubules. Unbound beads were then washed out and the number of beads binding to microtubules per microscope field were counted. Beads in >100 fields were counted per assay. Subsequently, the number of bound beads which moved in response to the uncaging of ATP was counted for each assay. The response to ATP of >20 beads were monitored per assay. CBF3 protein was purified as Fig. 3. The microtubule-motor activity of >30 beads were assayed per condition.

towards the plus-end, dynein towards the minus-end)¹³. The microtubules used in the assay were constructed so that their polarity could be distinguished directly under the microscope by virtue of a brighter minus-end (arrowhead in Fig. 2a). We observed more than 100 beads from four different CBF3 preparations, and saw only minus-end-directed movement distributed over a range of velocities (Fig. 2e).

To determine whether the motor activity was dependent on the presence of the CBF3 complex of proteins (Fig. 1a, lane WT), we assayed the microtubule-binding and motor activity of protein eluted from each affinity column. Very little DNA binding is observed from proteins eluted from the RN2011 affinity column⁵ (data not shown). Therefore if the microtubule motor activity is part of the CBF3 complex, very little motor activity should be seen in protein eluting from the RN2011 affinity column. To quantify the microtubule binding activity of the beads, we measured the number of beads bound to microtubules (per microscope field) and the number of these bound beads that moved along microtubules after ATP release. The results, shown in Table 1, demonstrate that protein prepared from the RN2011 mutant CEN DNA column (Fig. 1a, lane M) has greatly reduced microtubule binding and motor activity compared with protein eluted from a wild-type CEN DNA affinity column (lane WT). Microtubule-motor activity elutes only from an affinity column containing wild-type CEN DNA sequences, hence the motor is part of the CBF3 complex.

To confirm the specificity of the bead-motor interaction, we performed competition experiments with mutant and wild-type CEN DNA. We investigated whether soluble wild-type CEN DNA competes with bead-bound CEN DNA for microtubule-motor activity of the CBF3 complex. Binding reactions contained CBF3, wild-type CEN DNA beads and different amounts of soluble wild-type CEN DNA. The activity of the CBF3 beads was measured as a function of the amount of added soluble competitor. Between 1 and 10 fmol of added DNA reduced the number of active beads per field by 75% (Fig. 2f). Similar

TABLE 2 Movement of the CBF3 motor in response to different ATP analogues

ATP analogue	Velocity ($\mu\text{m min}^{-1}$)	% ATP velocity		
		CBF3	Kinesin	Dynein
ATP	4.04	100	100	100
GTP	0.6	15	27 ^a	0
Dimethyl ATP	3.9	96	20	0
Etheno ATP	1.7	42	13	0
PRTP	1.6	40	1.8	0
8-Bromo ATP	0.79	19	3.1	0

Analogues were used at 1 mM. Kinesin and dynein data were taken from ref. 14. Methods described in Fig. 2 legend.

binding reactions using the mutant RN2011 CEN DNA as a soluble competitor showed little effect on the microtubule-motor activity of the beads (Fig. 2f). Beads with RN2011 CEN DNA competed weakly with a radioactive wild-type CEN DNA fragment for CBF3 binding (Fig. 1c). These beads when incubated with CBF3 proteins should have a greatly reduced microtubule-binding activity. CBF3 proteins were incubated with beads that had either wild-type or RN2011 CEN sequences attached. The results (Table 1) show that the number of functional complexes per field seen with the RN2011 CEN DNA beads is only 3.0% of that obtained with wild-type CEN DNA beads (Table 1).

The nature of the motor protein in the CBF3 complex is not yet known. As described previously, there are three main proteins in the complex, but other less abundant proteins exist (Fig. 1a), thus it is unclear which proteins are responsible for the DNA binding, or motor activity. Currently, the main proteins are being cloned and sequenced, and DNA sequence homology with known microtubule motors may provide some clues. We compared the CBF3 motor with known motors, testing the response to a group of ATP analogues that are used with different efficiencies by the dynein and kinesin ATPases (Table 2)¹⁴. One feature that differentiates between the two families is that kinesin is able to use more ATP analogues to drive movement than dynein. Table 2 shows that the CBF3 motor was able to move with ATP analogues that can support kinesin but not dynein movement, indicating that the CBF3 motor may be related to the kinesin family of ATPases.

We have partially reconstructed kinetochore activity *in vitro* by showing that a complex of proteins that binds specifically to the *S. cerevisiae* centromere will link CEN DNA to microtubules *in vitro*. The microtubule-binding activity of the CBF3 complex seems to be mediated in part by microtubule-based motors. Such motors have been localized to kinetochores in higher eukaryotic spindles but their role in kinetochore function is unclear^{8,9}. Our results suggest that in *S. cerevisiae*, microtubule-based motors are an essential part of the DNA-microtubule linkage. How the activity of the motor is regulated will aid understanding of the mechanisms of chromosome movement at mitosis.

The movement of the CBF3 motor indicates that it may be involved in movement of chromosomes towards the minus-ends of microtubules at anaphase A (refs 15, 16). Anaphase A is defined in higher eukaryotes as the movement of the separated sister chromatids to opposite poles, and a similar movement seems to take place in *S. cerevisiae*^{17,18}. The velocity and direction of movement of the CBF3 motor make it an ideal candidate for an anaphase A motor in *S. cerevisiae*. But it is likely that, even in the apparently simple *S. cerevisiae*, there are other kinetochore activities that interact with microtubules *in vivo*. □

Received 26 March; accepted 11 August 1992.

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ACKNOWLEDGEMENTS. A.A.H. and K.M. contributed equally to this work. We are grateful to H. Lechner for introduction to the CBF3 preparation and for helpful discussion. We thank P. Sorger for help with molecular biology, T. Shimizu for help with ATP analogue experiments, and L. Cramer, R. Deshaies, A. Murray, K. Sawin, T. Stearns, V. Gelfand and P. Peluso for critical reading of the manuscript. A.A.H. is a Lucille P. Markey Scholar; J.C. is an American Cancer Society professor; and J.C. and T.J.M. are funded by grants from the NIH and the Packard and Searle foundations.

CENP-E is a putative kinetochore motor that accumulates just before mitosis

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THE mechanics of chromosome movement, mitotic spindle assembly and spindle elongation have long been central questions of cell biology¹. After attachment in prometaphase of a microtubule from one pole, duplicated chromosome pairs travel towards the pole in a rapid but discontinuous motion^{2,3}. This is followed by a slower congression towards the midplate as the chromosome pair orients with each kinetochore attached to the microtubules from the nearest pole. The pairs disjoin at anaphase and translocate to opposite poles and the interpolar distance increases. Here we identify CENP-E as a kinesin-like motor protein (M_r 312,000) that accumulates in the G2 phase of the cell cycle. CENP-E associates with kinetochores during congression, relocates to the spindle midzone at anaphase, and is quantitatively discarded at the end of the cell division. CENP-E is likely to be one of the motors responsible for mammalian chromosome movement and/or spindle elongation.

To identify novel proteins of the human centromere-kinetochore complex, we generated monoclonal antibodies against fractionated chromosomal proteins and used immunofluorescence to isolate antibodies that identify antigens localized at the centromeres. One such monoclonal antibody (177) identified CENP-E, a protein of M_r 312K that could not be detected in interphase cells, but first appeared at the centromere regions of chromosomes in prometaphase⁴. To determine the primary structure of CENP-E, antibody 177 was used to screen a human complementary DNA expression library. A 2-kilobase (kb) cDNA (clone P2, Fig. 1a) identified an ~8 kb HeLa cell RNA (data not shown), large enough to encode CENP-E. To verify whether this partial cDNA encoded a portion of CENP-E, a rabbit polyclonal antiserum (pAb1) was raised against a bacterially produced protein encoded by a subclone of the P2 cDNA (stippled domain in Fig. 1a) that did not encode the antibody 177 epitope. On immunoblots of chromosomal proteins this antiserum identified a high-molecular-mass protein, whose size was indistinguishable from the antigen identified using antibody

177 (Fig. 2a), and which stained the centromere region of isolated chromosomes (Fig. 2b). The polyclonal antiserum also revealed (Fig. 2c) the same cell cycle-dependent localization of the antigen in HeLa cells as was seen previously with antibody 177 (ref. 4). The recognized antigen was localized to a set of punctate dots on prometaphase and metaphase chromosomes. At anaphase, the antigen became detached from chromosomes and localized exclusively to fibres in the spindle midzone and to the midbody during telophase. We conclude that this cDNA clone is derived from a portion of the CENP-E messenger RNA.

By rescreening the original cDNA library, and by construction of a sub-library primed by a CENP-E-specific oligonucleotide, a set of eight overlapping cDNAs which spanned a total of 8.3 kb were obtained (Fig. 1a). DNA sequencing revealed a composite open reading frame (encoding 2,663 amino acids)

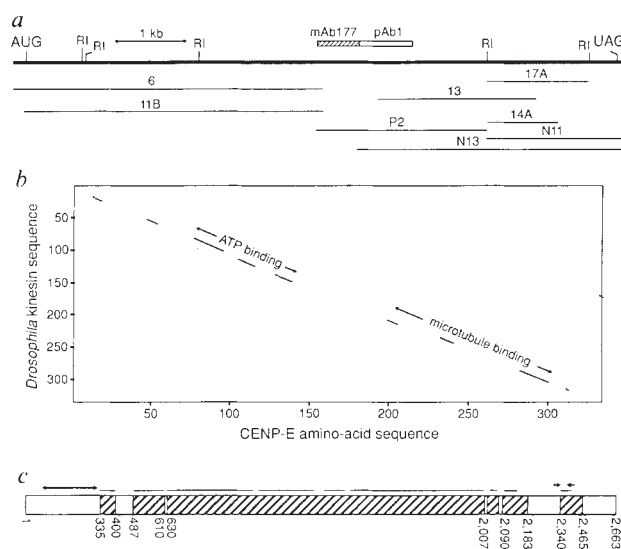


FIG. 1 Schematic diagram of CENP-E cDNA clones, comparison of the CENP-E and kinesin heavy-chain motor domains, and predicted structure of the CENP-E polypeptide. **a**, Schematic drawing of eight overlapping cDNA clones that span the 8,371-base CENP-E messenger RNA. Positions of translation initiation (AUG) and termination (UAG) and the putative polyadenylation signal (pA) are noted. RI, positions of *EcoRI* sites. Stippled box denotes the domain used to generate pAb1, a rabbit polyclonal antibody against bacterially expressed CENP-E. The cross-hatched domain denotes the region that contain the antibody 177 epitope. **b**, Dot matrix homology plot (Macvector, [B]) comparison of the motor domains (335 amino acids) of *Drosophila* kinesin heavy-chain and CENP-E. The portions of *Drosophila* kinesin that represent its known ATP and microtubule binding domains¹⁰ are noted. **c**, Predicted secondary structure of the CENP-E polypeptide. Cross-hatched domains are predicted to adopt α -helical structures⁵. Overlined domains are predicted to adopt coiled-coil conformations⁶. Arrows above denote regions of >50% sequence identity to *Drosophila* kinesin heavy chain.

METHODS. Plaques (5×10^5) from a λ gt11 human breast carcinoma cDNA library (Clontech) were probed with antibody 177 after induction of fusion protein expression with isopropyl- β -D-thiogalactoside. Immunopositive clones were identified with ¹²⁵I-labelled goat anti-mouse antibodies (Amersham). After plaque purification, the cDNA from the initial cDNA clone (P2) was used to rescreen the same library. To extend the P2 cDNA towards the 5' end of the mRNA, an oligonucleotide corresponding to the sequence near the 5' end of P2 was used as a primer for reverse transcription of poly(A)⁺ RNA obtained from HeLa cells synchronized in G2. Synthesis of cDNA was done using a kit (Pharmacia) and after addition of *NotI*/*EcoRI* adaptors, the cDNA was ligated into *EcoRI*-digested ZAPII and packaged into phage particles (Stratagene). Unamplified plaques (10^5) were screened using a 5' portion of the P2 cDNA. The authenticity of each new clone was initially verified by its ability to identify the same 8-9 kb RNA on RNA blot analysis. Subsequently antibodies raised against bacterially derived fusion proteins encoded by each new cDNA portion were shown to recognize CENP-E.

preceded by in-frame translation terminators (Fig. 3). The predicted CENP-E polypeptide structure⁵ consists of three domains: amino- and carboxy-terminal globular domains, each of ~500 amino-acid residues, separated by a central 1,700 amino-acid segment which may form a discontinuous α -helix (Fig. 1c). This helical domain contains an extensive, hydrophobic heptad repeat characteristic of coiled-coil helices⁶. Comparison of the sequence of CENP-E with all sequences in current gene and protein data bases revealed that the N-terminal 335 amino acids share extensive homology with the motor domain of all known kinesin-like proteins⁷⁻¹⁶. Within this motor region, CENP-E shares virtual sequence identity (Fig. 1b) with the 120 residues that comprise the highly conserved nucleotide- and microtubule-binding sites characteristic of the kinesin family of proteins¹⁷. In sequence and in predicted structure, CENP-E is most like the conventional kinesin heavy-chain, although its α -helical 'rod' segment is nearly four times longer than that of kinesin. Unlike other kinesin-like proteins, sequence comparison revealed an additional 18-amino-acid segment near the C terminus of CENP-E (Fig. 1c; residues 2,343-2,360) with 50% sequence identity (78% if highly conservative substitutions are counted) to sequences in the C termini of heavy-chain kinesins^{10,11,15,16}. As kinesin light-chains bind in this globular tail region¹⁸, this conserved domain may represent a binding site for kinesin light-chains or a related accessory factor(s).

That CENP-E seems to be absent from ~90% of interphase cells (for example, arrowed cells in bottom panels of Fig. 2c; see also ref. 4) suggests that the abundance of CENP-E might be dependent on the phase of the cell cycle. To test this directly, HeLa cells were synchronized at the G1/S boundary and after release, extracts from equal numbers of cells at various points

in the cycle were examined for accumulated CENP-E. Cells in the G1 and early S phases showed little detectable CENP-E in either cytoplasmic or nuclear fractions, but cytoplasmic CENP-E levels rose sharply during late S and G2/M (Fig. 4a, upper panel), ultimately reaching the level found in cells arrested in prometaphase by inhibition of spindle assembly. At all times CENP-E is excluded from nuclei (Fig. 4a, lower panel), consistent with the absence of a consensus nuclear targeting signal¹⁹⁻²¹.

To examine the loss of CENP-E at the end of mitosis, CENP-E synthesized during late S phase was pulse-labelled and followed during the subsequent cell cycle. After 1 h, when most cells were still in G2 or had just entered mitosis, CENP-E synthesized earlier remained stable (Fig. 4b). When most cells had completed mitosis, only ~20% of CENP-E synthesized in the preceding G2 phase remained. This loss of CENP-E was dependent on completion of mitosis, as CENP-E remained stable in cells blocked in mitosis with colcemid. Even after a 4 h chase, when virtually all CENP-E was lost from cells that had completed mitosis, CENP-E remained in colcemid-arrested cells (Fig. 4b).

The ability of CENP-E to interact with microtubules in HeLa cell extracts was tested. CENP-E quantitatively sedimented after taxol-stimulated microtubule assembly (Fig. 4c, lane 2), but not when microtubule assembly was blocked by the addition of colcemid (Fig. 4c, lane 3). As with other kinesin-like proteins, CENP-E still co-sedimented with microtubules after the addition of 1 mM β - γ -imidoadenosine 5'-phosphate (AMP-PNP), an inactive ATP analogue, or depletion of ATP (Fig. 4d, lanes 6, 9). But addition of 10 mM ATP released only ~50% of microtubule-bound CENP-E (Fig. 4d; compare lanes 10 and

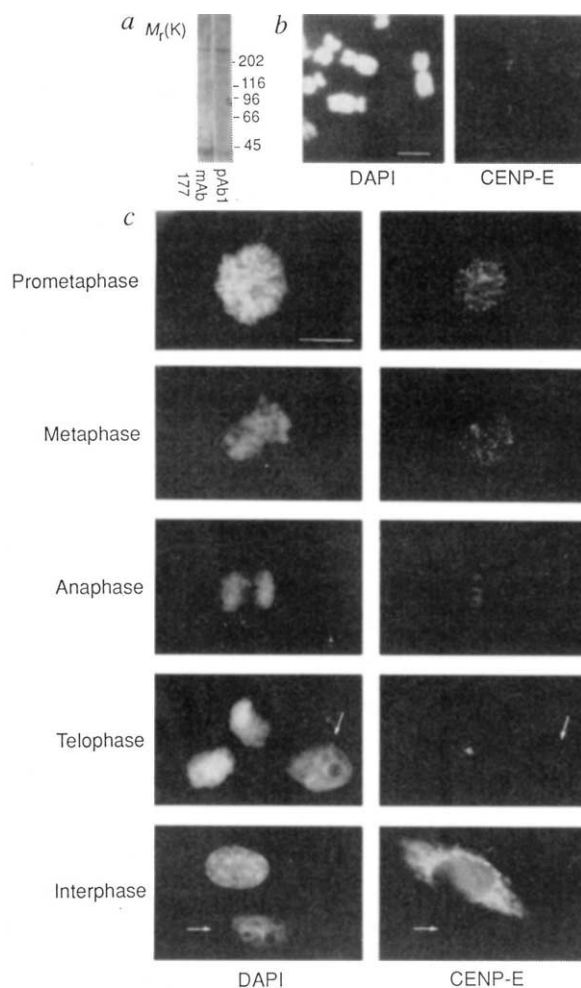


FIG. 2 Authentication of CENP-E cDNA clones. *a*, Immunoblotting of HeLa chromosomal proteins using (lane 1) pAb1 and (lane 2) antibody 177, a polyclonal antibody generated against a portion (stippled domain in Fig. 1a) of the P2 cDNA that does not encode the antibody 177 epitope. Migration position of myosin (M_r 202K) is shown to the left. *b* (left), DAPI stain of chromosomes and (right) indirect immunofluorescence localization of CENP-E at the centromeres of isolated HeLa chromosomes using pAb1. Scale bar, 5 μ m. *c*, Indirect immunofluorescence localization of CENP-E in HeLa cells at various points in the cell cycle and visualized using pAb1. Scale bar, 10 μ m. Arrows in lower panels mark interphase cells that do not stain for CENP-E.

METHODS. Chromosomal proteins were prepared from HeLa cells, immunoblotted⁴ and then incubated either with antibody 177 or pAb1, a polyclonal antibody generated against a fusion protein made up of the bacterial protein trpE linked in frame with the polypeptide encoded by the 863-bp *Xho*I-*Hind*III fragment of cDNA clone P2 (stippled region in Fig. 1a). Primary antibody was detected with ¹²⁵I-labelled secondary antibodies or protein A. To produce pAb1, the P2-encoded protein, expressed using the pATH plasmid vectors and the protease-deficient *Escherichia coli* strain CAG456, was gel-purified and rabbits were immunized. Indirect immunofluorescence staining of isolated HeLa chromosomes and cells was done as described⁴, except that rabbit polyclonal antibodies (*a*) were used as the primary antibody, followed by biotinylated anti-rabbit antibodies (Vector) and streptavidin conjugated with Texas Red (Sigma). Slides were examined on a Zeiss Axiophot microscope equipped with epifluorescence optics and images photographed using Kodak Tmax 400 film.

538

[illegible]

FIG. 3 Nucleotide and deduced amino-acid sequence of CENP-E. Composite nucleotide and predicted amino-acid sequence of CENP-E were determined by sequencing the overlapping cDNAs shown in Fig. 1a. Underlined sequences are predicted⁶ to form an α -helical, coiled-coil conformation. All regions were sequenced on both strands using the chain-termination method.

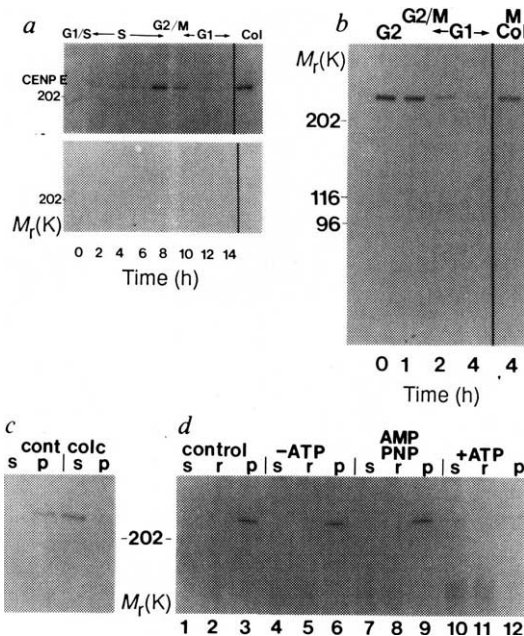


FIG. 4 Cell cycle-dependent accumulation and loss, and microtubule binding of CENP-E. *a*, Steady-state levels of CENP-E in the cytoplasm (upper panel) and nuclei (lower panel) of cells at different stages of the cell cycle, marked above. Col, cells blocked in mitosis by addition of colcemid. *b*, The turnover of CENP-E at the end of mitosis observed by pulse-chase and immunoprecipitation. *c*, Cosedimentation of CENP-E with microtubules assembled in HeLa extracts of control (cont) and colcemid-treated (colc) cells. *d*, Nucleotide

sensitivity of the interaction between CENP-E and microtubules; s, supernatant; r, rinse; p, pellet.

METHODS. *a*, HeLa cells grown in suspension were incubated in excess thymidine (2.5 mM). The cells were released into complete media for 9 h and aphidocolin (Calbiochem) was added to a final concentration of $5 \mu\text{g ml}^{-1}$ to synchronize cells at the G1/S boundary. Cells were collected every 2 h after release from the block and cell synchrony was monitored by flow cytometry. To measure the steady-state levels of CENP-E, synchronized cells were lysed in 2 ml of RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.5% deoxycholate, 1.0% NP-40, 0.1% SDS, 1 mM phenylmethylsulphonyl fluoride (PMSF), $1 \mu\text{g ml}^{-1}$ each of leupeptin, aprotinin and pepstatin) and centrifuged at $15,000g$ for 20 min. The pelleted nuclei and insoluble debris were boiled in SDS-sample buffer, separated by electrophoresis and transferred onto Immobilon P (Millipore) for immunoblot analysis. Cytoplasmic extracts were incubated with pAb1 (1:200 final) at 4°C for 2 h. A 1:1 slurry (40 μl) of washed Protein A Sepharose (Pharmacia) beads were added and the immune complex was washed in RIPA buffer, boiled in SDS-sample buffer, separated on a 4–10% gradient gel, transferred onto the membrane and processed for immunoblot analysis using the same antibody that was used for the immunoprecipitation (used at a 1:1,000 dilution). ^{125}I -labelled protein A (ICN) was used to detect bound primary antibodies. For pulse-chase experiments, cells in G2 were radiolabelled for 1 h using Trans-label (ICN) at a final concentration of $100 \mu\text{Ci ml}^{-1}$. After labelling, an aliquot of cells was immediately lysed. The remainder were released into complete media until the end of mitosis. To block cells in mitosis, cells were chased in unlabelled media that contained $0.5 \mu\text{g ml}^{-1}$ of colcemid (Gibco). Cell lysates were processed for immunoprecipitation as described. For analysis of the sedimentation of CENP-E with microtubules, 1×10^9 HeLa cells were pelleted, washed, and the packed cell pellet was resuspended in an equal volume of homogenization buffer (80 mM PIPES, pH 6.9, 1 mM MgSO_4 , 1 mM EGTA, 0.1 mM dithiothreitol, 1 mM PMSF and $1 \mu\text{g ml}^{-1}$ each of leupeptin, aprotinin and pepstatin). An S-100 extract was incubated with taxol ($10 \mu\text{M}$ final) to induce microtubule polymerization. Microtubules were pelleted by centrifugation at $100,000g$. Supernatants were saved and the pellets were rehomogenized in taxol-containing buffer and recentrifuged as described. To detect CENP-E, the supernatants were combined and adjusted to $1 \times \text{RIPA}$ buffer. The pellets were rinsed with 1 ml of polymerization buffer and directly solubilized with RIPA buffer. CENP-E was immunoprecipitated and processed for immunoblot analysis as described above. For ATP depletion, 20U ml^{-1} hexokinase and 20mg ml^{-1} glucose were added to the S-100 extracts for 15 min before inducing microtubule polymerization. In some experiments, AMP-PNP or Mg-ATP was present during the polymerization reaction at final concentration of 1 mM or 10 mM, respectively. To block microtubule assembly, colcemid (final concentration $1 \mu\text{g ml}^{-1}$) was added to the S-100 extracts.

12), similar to the situation found previously for the *non-claret disjunctional* (*ncd*) protein¹². The nucleotide-dependent partial release of CENP-E indicates that CENP-E might interact with microtubules directly or indirectly through a nucleotide-insensitive site. This is supported by the fact that the C-terminal 95 amino acids that comprise a basic (pI 9.3), proline-rich (13.4%) domain, similar to the putative microtubule binding sites of tau and MAP2 (ref. 22).

What is the role of CENP-E during mitosis? The striking cell cycle-dependent accumulation and loss of CENP-E, and the

presence of CENP-E at kinetochores during chromosome congression and at the midzone during spindle elongation, give strong support to the view that CENP-E is an important motor molecule in chromosome movement and/or spindle elongation. Consistent with this, microinjection of one CENP-E antibody transiently arrests cells at metaphase⁴. Further experiments to demonstrate motor activity, to determine the direction of CENP-E mediated movement, and to inhibit CENP-E function *in vivo* are now needed to document more clearly the role(s) of CENP-E. □

Received 22 April; accepted 27 August 1992.

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ACKNOWLEDGEMENTS. We thank L. Wordeman for advice in the microtubule binding assay. Taxol was a gift from the National Cancer Institute. This work has been supported by grants from the L. P. Markey Foundation to T.J.Y. and NIH to D.W.C. B.T.S. was supported by a pre-doctoral NIH training grant.

Mitotic spindle organization by a plus-end-directed microtubule motor

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INTRACELLULAR microtubule motor proteins^{1,2} may direct the motile properties and/or morphogenesis of the mitotic spindle (reviewed in ref. 3). The recent identification of kinesin-like proteins important for mitosis or meiosis⁴⁻⁹ indicates that kinesin-related proteins may play a universal role in eukaryotic cell division, but the precise function of such proteins in mitosis remains unknown. Here we use an *in vitro* assay for spindle assembly, derived from *Xenopus* egg extracts^{10,11}, to investigate the role of Eg5, a kinesin-like protein in *Xenopus* eggs¹². Eg5 is localized along spindle microtubules, and particularly enriched near spindle poles. Immunodepletion of Eg5 from egg extracts markedly reduces the extent of spindle formation in extracts, as does direct addition of anti-Eg5 antibodies. We also demonstrate that Eg5 is a plus-end-directed microtubule motor *in vitro*. Our results suggest a novel mechanism for the dynamic self-organization of spindle poles in mitosis.

The Eg5 protein is predicted to consist of three domains: a globular amino-terminal 'head' or motor domain containing the sequences shared among kinesin-like proteins, an α -helical 'stalk' domain that is expected to form a coiled-coil, and a globular carboxy-terminal 'tail'¹². Polyclonal antibodies against the non-conserved stalk domain of Eg5 were generated using

FIG. 2 Eg5-dependent microtubule motility. *a*, Portions of a video sequence showing plus-end-directed movement of microtubules over time, indicated in seconds. Scale bar, 10 μ m. *b*, Distribution of mean velocities of 40 microtubules, chosen at random from the video record.

METHODS. GT-Eg5HS was purified as described in Fig. 1. Motility assays using polarity-marked fluorescent microtubules¹⁵ were performed by binding purified protein (0.5–1 mg ml⁻¹ in Na-BRB80 (80 mM Na-PIPES, pH 6.8, 1 mM MgCl₂, 1 mM EGTA) in a flow cell and then flowing in casein (5 mg ml⁻¹ in 50 mM K-HEPES, pH 8.0) and taxol-stabilized, polarity-marked microtubules. After extensive washing with motility buffer (Na-BRB80, 50 mM NaCl, 10 μ M taxol), microtubule gliding was activated by flowing in motility buffer containing 10 mM Mg-ATP and an enzymatic anti-photobleaching system¹⁵. Video sequences of gliding microtubules were recorded with a silicon-intensified target camera onto optical disk, and measurements of velocity taken semi-automatically, using a Maxvision image processing system (Datacube) and home-written software²¹.

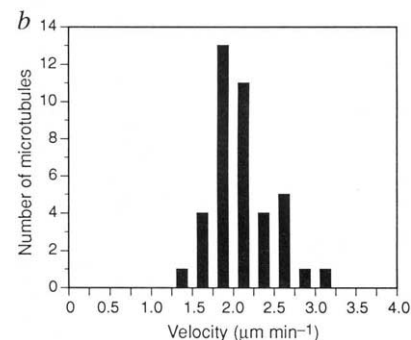
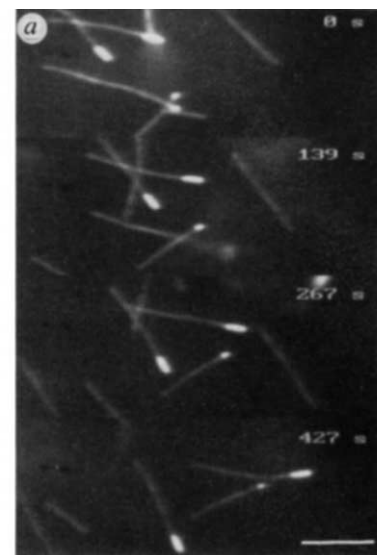
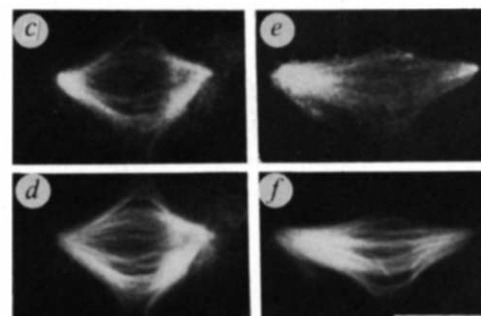
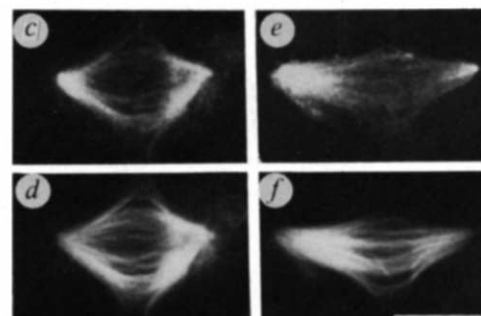
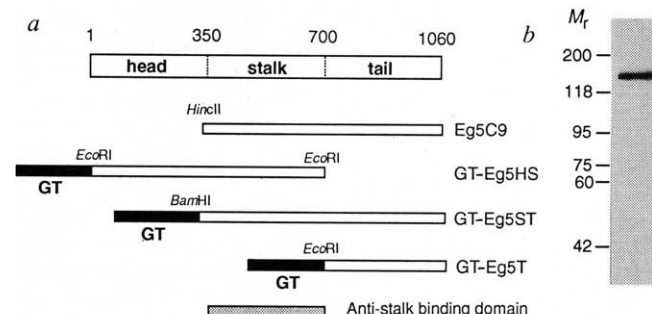


FIG. 1 Generation of specific antibodies to the Eg5 stalk domain. *a*, Schematic diagram of the Eg5 protein. Approximate position of amino-acid residues flanking predicted domain boundaries are shown above. Constructs used in this work, and the domain of Eg5 to which anti-stalk antibodies bind, are shown below. The glutathione S-transferase domain (GT) of fusion proteins is shown in black. *b*, Immunoblot of total *Xenopus* egg extract, stained with anti-stalk antibody. *c–f*, Immunofluorescent staining of Eg5 (*c, e*) in two metaphase spindles assembled *in vitro*, compared with corresponding total-tubulin staining (*d, f*). Scale bar is 20 μ m.

METHODS. *a*, Constructs were generated from a novel complementary DNA clone (to be described elsewhere) which is 93% identical (>95% highly conserved residues) to the original Eg5 cDNA and which is probably allelic to Eg5 because of pseudotetraploidy in *Xenopus laevis*. The C-terminal fragment Eg5C9 was subcloned into pET-3cp (refs 33, 34) and induced in *Escherichia coli* BL21(DE3). The insoluble fraction was purified by SDS-PAGE and injected into rabbits. To generate GT fusion proteins, the N-terminal fragment Eg5HS and the small C-terminal fragment Eg5T were subcloned into the *Eco*RI site of pGEX 1–1 (ref. 35). The large C-terminal fragment Eg5ST was subcloned into the *Bam*HI site of pGEX-3X. Fusion proteins were induced in *E. coli* NB42 with 50 μ M isopropyl- β -D-thiogalactoside for 4 h at 25 °C and purified on glutathione-agarose (D. Kellogg, personal communication). *b*, Anti-stalk antibodies were affinity-purified by passing anti-C9 antisera over a column of GT-Eg5HS coupled to Affigel-10 (Biorad), and washing with non-ionic detergent and high salt before elution with 100 mM glycine, pH 2.0. The affinity-purified antibodies were judged to be >95% pure IgG by Coomassie staining. A similar approach yielded antibodies specific for the tail domain, which reproduced all of the results described here (not shown). Total crude *Xenopus* egg extract was separated by SDS-PAGE, transferred to nitrocellulose, and stained with anti-stalk antibody at 5 μ g ml⁻¹. Blots were developed using the ECL kit (Amersham). *c*, Spindles were assembled in crude *Xenopus* egg extracts as described^{11,36}, using tetramethylrhodamine-labelled tubulin as a tracer at 0.3 mg ml⁻¹ and demembrated sperm nuclei at a final density of 100–200 per μ l. Spindles were prepared for immunofluorescence as described¹¹, with slight modifications. Anti-stalk antibodies were used at 10 μ g ml⁻¹. Staining was



specifically competed by preincubating antibody with fusion proteins containing the Eg5 stalk domain, but not by other control proteins (data not shown). Series of optical sections of spindles were collected with a Biorad MRC-600 laser scanning confocal microscope in dual-label mode and projected onto a single image plane using Biorad software. All contrast manipulations and enhancements were strictly linear.

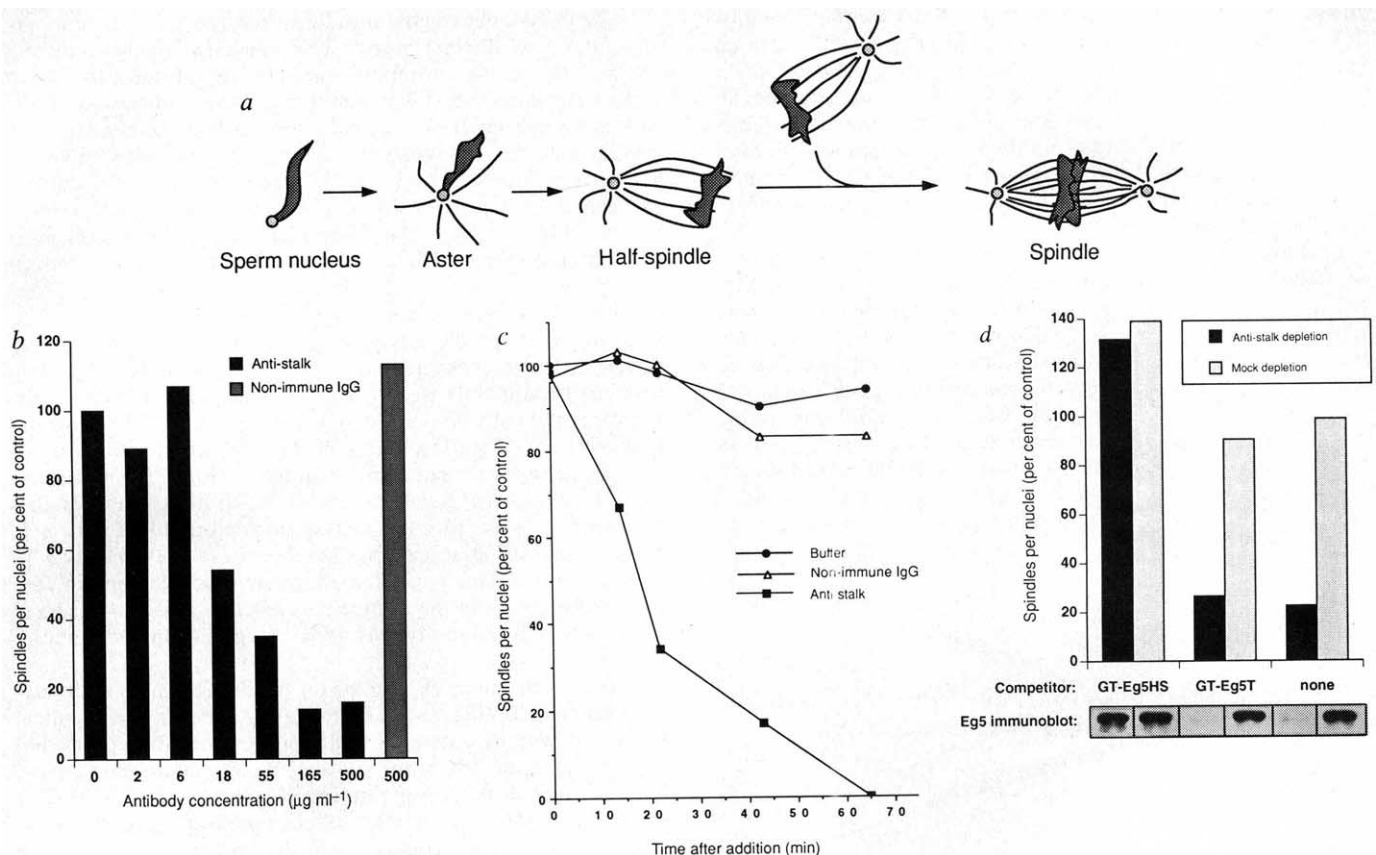


FIG. 3 Inhibition of spindle assembly by anti-stalk antibodies. **a**, Pathway of spindle assembly in metaphase-arrested *Xenopus* egg extracts. **b**, Dose-dependence of inhibition of spindle assembly by anti-stalk antibodies. **c**, Disruption of pre-existing spindles by anti-stalk antibodies. **d**, Immunodepletion of Eg5 from extracts using performed complexes that competed with different Eg5 fusion proteins, shown with correlate levels of spindle assembly. Levels of Eg5 remaining in extract supernatants after 'depletion' are shown below. **e-h**, Microtubule arrays in extracts formed in the presence of control IgG (**d, f**) and anti-stalk antibodies (**e, g**), at 20 min (**e, f**) and 45 min (**g, h**). Rhodamine-labelled microtubules are red, and chromatin is green. Scale bar is 20 μm (**e, f, g**); 30 μm (**h**).

METHODS. **b**, Anti-stalk antibodies were dialysed against, and diluted serially in 50 mM potassium glutamate and 0.5 mM MgCl_2 before addition to 10 volumes of extract. After 1 h incubation at 4 $^{\circ}\text{C}$, sperm nuclei were added and spindle formation assayed as in Fig. 1. Purified non-immune rabbit IgG was used as a control. The extent of spindle assembly was assayed by fixing extracts *in situ*^{11,36} after 40–60 min incubation and counting the number of spindles present as a percentage of total sperm nuclei. Over 200 nuclei were counted for each data point. In typical controls, 25–40% of nuclei give rise to spindles, depending on the extract. **c**, Anti-stalk antibody ($165 \mu\text{g ml}^{-1}$) was added to spindles already formed in extracts after incubation with sperm nuclei for 60 min. After antibody addition aliquots of extracts were scored for total spindle number as described above. **d**, Preformed complexes of Protein A Affiprep beads (Biorad) with anti-stalk antibodies or control IgG were incubated with either GT-Eg5HS, GT-Eg5T (purified as above), or no additional protein. After washing, the bead complexes were added to extracts as above and incubated for 2–3 h with rotation. Beads were pelleted as above and supernatants were used to assay spindle assembly, and levels of Eg5 remaining in the extracts by immunoblotting, as described in Fig. 1. **e-h**, Extracts were fixed *in situ* as described, except that the fixative contained 100 μM chromomycin A3 to stain DNA. Microtubules and chromatin were imaged using the confocal microscope as described in Fig. 1, and the two images were merged in pseudocolour, using Biorad software.

Disrupted half-spindles were scored as a percentage of total half-spindles. Rosettes were scored as 'monopolar' arrays (as in **h**) containing chromatin derived from three or more independent nuclei.

purified recombinant protein, and affinity-purified against different fusion proteins (Fig. 1*a, b*). Spindles assembled in an *in vitro* reconstitution system¹¹ were stained with anti-stalk antibodies, and Eg5 was found to localize to microtubules in the spindle (Fig. 1*c, e*). Careful examination revealed that Eg5 staining is enriched towards spindle poles, compared with total tubulin staining (Fig. 1*d, f*). Similar patterns of Eg5 staining in mitosis are observed in mammalian tissue-culture cells (cell line PtK2; not shown).

To determine whether Eg5 can act as a microtubule motor, we tested an Eg5 head-stalk fusion protein (GT-Eg5HS; Fig. 1*a*) in an *in vitro* motility assay^{13,14}. Microtubule polarity was marked with a single, brightly fluorescent seed near the minus ends of microtubules¹⁵. Microtubules moved on addition of ATP, led by the seed ($n > 100$), indicating that Eg5-driven motility is plus-end-directed (Fig. 2*a*). Under optimal conditions translocation occurred at an average of $2.1 \mu\text{m min}^{-1}$ (s.d. = 0.36; Fig. 2*b*). This is slow compared with translocation by kinesin and *ncd* ($30\text{--}40 \mu\text{m min}^{-1}$ (ref. 14) and $4\text{--}15 \mu\text{m min}^{-1}$ (refs 16, 17), respectively) and suggests that Eg5 is also a slow motor *in vivo*.

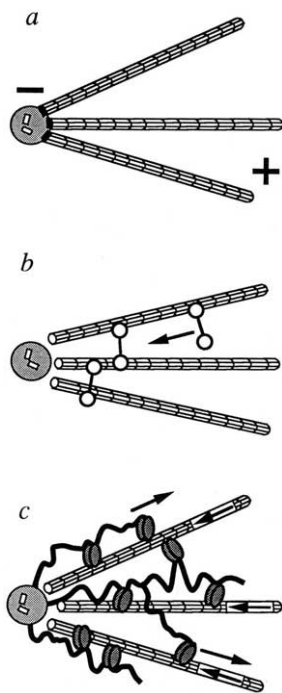


FIG. 4 Multiple modes of spindle pole organization in the dynamic steady state. Microtubule minus ends (–) are shown proximal to the centrosome, and plus ends (+) distal. *a*, Microtubules are anchored through their minus ends to the centrosomal nucleating site. This static 'textbook' picture must be modified to account for poleward microtubule flux in the spindle^{19–21}. *b*, Microtubules are released from centrosomal nucleation sites after nucleation. But they can be bundled and organized to some extent by microtubule-associated proteins, and in particular minus-end-directed motors such as cytoplasmic dynein³⁷, shown here schematically as a two-headed molecule, crosslinking microtubules and moving towards their minus ends. Although contributing to the formation of bundled microtubule arrays and to overall spindle structure, this level of organization would not be sufficient to 'centralize' the spindle pole as a coherent, focused structure, especially as microtubules are released from the centrosome. Failure to focus the pole would result in the type of structure seen in Fig. 3*f*. *c*, The morphogenetic organizing activity proposed for the Eg5 plus-end-directed motor. Eg5 (double ovals) is bound to microtubules by its motor domain but tethered to a centrosomal filament system (thick wavy lines) by its tail domain. Eg5 activity extends the filaments towards microtubule plus-ends. The free arrows in *b* and *c* represent the vectorial movement of motor proteins. The arrows embedded in microtubules indicate poleward microtubule flux, which could be driven by the Eg5 microtubule motor.

In *Xenopus* egg extracts, spindle morphogenesis can be separated into two distinct steps¹¹ (Fig. 3*a*). In the first, factors intrinsic to mitotic chromatin stabilize microtubules locally to form an asymmetric, polar microtubule array, the half-spindle. In the second step, these half-spindles fuse head-to-head to form bipolar spindles. Preincubation of egg extracts with anti-stalk antibody before assaying spindle assembly markedly reduced the extent of bipolar spindle assembly in a dose-dependent manner (Fig. 3*b*). Moreover, the addition of antibody to extracts containing preassembled spindles also led to the efficient disruption of spindles (Fig. 3*c*). Inhibition of spindle assembly correlated with changes in morphology of microtubule arrays. At early stages of spindle assembly (15–20-min incubation), mock-treated extracts contained stereotyped half-spindles (Fig. 3*e*), whereas in anti-stalk-treated extracts the poles of many half-spindles (typically 45–70%, compared with 1–10% in controls) were splayed (Fig. 3*f*). After 40–60 min, when mock-treated extracts formed normal bipolar spindles (Fig. 3*g*), anti-stalk-treated extracts contained unusual rosette-like structures that appeared to form from the linking of microtubule minus-ends of disrupted half-spindles (Fig. 3*h*). Rosettes were typically 5–10 times more numerous in anti-stalk-treated extracts than in controls. An increase in the numbers of rosettes was also observed when anti-stalk antibody was added to preassembled spindles (data not shown).

These effects are unlikely to be the result of bivalent antibody-induced crosslinking, as IgG and Fab fragments had identical effects on spindle assembly, half-spindle pole disruption and rosette formation (data not shown). We also immunodepleted Eg5 from extracts, using preformed complexes of anti-stalk antibodies and protein A beads. Preformed complexes that were incubated with stalk-containing fusion proteins before being added to extracts were rendered incapable of Eg5 depletion, whereas complexes preincubated with control proteins depleted Eg5 (Fig. 3*d*). Depletion of Eg5 was correlated with inhibition of spindle assembly. Immunodepleted extracts also showed defects in half-spindle pole organization (data not shown).

Spindles formed in the aforementioned assay normally do not contain kinetochores or kinetochore fibres¹¹. We therefore investigated whether the added complexity of kinetochores and kinetochore microtubules could compensate for the loss of Eg5 function, using egg extracts that undergo a single round of DNA replication before entering mitosis^{11,18}. Again, in the presence of anti-stalk antibody ($165 \mu\text{g ml}^{-1}$), the level of normal stereotyped spindle assembly was inhibited by 70% (data not shown). Observed abnormalities were pleiotropic and included problems in chromosome congression, in the number and organization of spindle poles, in spindle shape, and combinations of these.

Poleward microtubule flux in the steady-state, dynamic metaphase spindle and also in the half-spindle (K.E.S., unpublished observations) specifically requires that microtubule minus-ends at spindle poles be labile to depolymerization as new tubulin subunits are incorporated at microtubule plus-ends^{19–21}. We propose that this lability is achieved by the release of microtubule minus-ends from their primary nucleation sites^{22–24} (Fig. 4*a, b*) and that a more lasting, global connection between the centrosome and released spindle microtubules is mediated by the Eg5 protein. According to this model, the Eg5 motor-domain binds to and generates force against microtubules, while either the stalk or tail domain tethers Eg5 to extensible filaments emanating from the centrosome (Fig. 4*c*). Proteins of this hypothetical 'motor filament system' are likely to be long, extended molecules containing centrosomal-targeting signals, perhaps forming larger-order polymeric networks. Such proteins could represent the hypothesized 'spindle matrix' proteins^{25–27}, a possible candidate being the recently cloned NuMA protein, which has extensive coiled-coil domains and is localized near the spindle poles^{28,29}. It is also possible that Eg5 itself might form filaments.

What is the relation between aberrant half-spindle poles and the failure to form bipolar spindles? We believe that Eg5 may be required primarily for spindle-pole organization. 'Downstream' events such as bipolarization of microtubule arrays would in this case be strongly affected by the absence of normal Eg5 activity, albeit indirectly, perhaps as a consequence of changes in microtubule dynamics. But it is possible that Eg5 also cross-bridges antiparallel microtubules from the two half-spindles. As microtubule dynamics in the spindle are complex, we see no reason to accept a direct cross-bridging function for Eg5, but as yet we have no strong evidence to rule it out. The limited formation of spindles in Eg5-depleted extracts would be consistent with factors other than Eg5, perhaps additional microtubule motors, mediating bipolarity.

Mutants in the fungal mitotic kinesin genes *bimC*, *cut7*, *CIN8* and *KIP1* (refs 4, 6, 30–32) seem not to form bipolar spindles

because of a failure in spindle pole separation, and one explanation offered for this phenotype is that these proteins mediate interactions between antiparallel microtubules^{30,31}. The motor domain sequences of these proteins are quite similar to the sequence of Eg5 (ref. 12). We believe our data from *Xenopus* egg extracts are consistent with those from analogous experiments in fungal genetics and cytology. We propose that the *bimC-cut7* family in fungi may also be involved in the functional organization of spindle poles, and that the failure to form a bipolar spindle may be an indirect consequence of the loss of this activity. We believe that the mechanism of Eg5 function in mitosis may be universally conserved.

Note added in proof: Immunoblotting and immunoprecipitation experiments (not shown) indicate that Eg5 is identical to LH-145, a kinesin-like microtubule-binding protein identified previously³⁸. □

Received 19 May; accepted 13 August 1992.

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ACKNOWLEDGEMENTS. This paper is dedicated to Daniel Mazia. We thank members of our respective laboratories and of the laboratory of M. Kirschner for advice and criticism. We also thank Andy Hoyt and Bill Saunders for sharing data before publication. This work was supported by the NIH, the David and Lucile Packard Foundation (T.J.M.), the NSF, the UCSF Chancellor's Office (K.E.S.) the EEC, INSERM and the Ligue d'Ille et Vilaine de Lutte contre le Cancer (M.P.).

A plus-end-directed motor enzyme that moves antiparallel microtubules *in vitro* localizes to the interzone of mitotic spindles

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MITOSIS comprises a complex set of overlapping motile events, many of which involve microtubule-dependent motor enzymes^{1,2}. Here we describe a new member of the kinesin superfamily. The protein was originally identified as a spindle antigen by the CHO1 monoclonal antibody³ and shown to be required for mitotic progression^{4,5}. We have cloned the gene that encodes this antigen and found that its sequence contains a domain with strong sequence similarity to the motor domain of kinesin-like proteins. The product of this gene, expressed in bacteria, can cross-bridge antiparallel microtubules *in vitro*, and in the presence of Mg-ATP, microtubules slide over one another in a fashion reminiscent of microtubule movements during spindle elongation.

We previously used the monoclonal antibody CHO1 to identify a novel protein component of mammalian mitotic spindles^{3–5}. To clone the gene that encodes this antigen, we screened a HeLa cell complementary DNA expression library with CHO1. Four immunopositive plaques were identified

among 800,000 screened. These all contained cDNA that cross-hybridized, shared overlapping restriction maps and hybridized with a 3.5-kilobase (kb) messenger RNA (Fig. 1a). Furthermore, in the regions where the clones overlap, their nucleotide sequences were identical, indicating that they were derived from the same gene (Fig. 1b). The largest of these clones spans 3.6 kb and contains a single open reading frame of 2,913 base pairs (bp) (Fig. 1c). Southern blot analysis suggests that the CHO1 antigen is encoded by a single gene (data not shown).

This open reading frame predicts a protein of 961 amino acids with a calculated molecular mass of 110,000 (110K). The amino-terminal half of the CHO1 antigen contains a region of 350 amino acids that shares significant identity (30–45%) with the motor domains of other members of the kinesin superfamily^{6,7}. Within this conserved region are several shorter regions (including the sequences LAGSE and SSHSR; single-letter amino-acid code)^{8,9} that are found in most kinesin-like proteins. But the carboxy-terminal half of the CHO1 antigen shows little homology with any other kinesin-like proteins, so this seems to be a novel member of the kinesin superfamily. Secondary structure analysis¹⁰ predicted that a central region between amino-acids 485 and 655 may form an α -helix¹¹. This region contains heptad repeats of hydrophobic residues and, like myosin and kinesin heavy chains, may assemble into a coiled-coil^{12,13}.

The predicted amino-acid sequence of the CHO1 antigen revealed a putative nuclear localization signal¹⁴ near its N terminus, as well as consensus phosphorylation sites for both p34^{cdc2} kinase and casein kinase II (Fig. 1c). These features are characteristic of nuclear proteins¹⁵ and are consistent with the observation that the CHO1 antigen is localized to interphase nuclei⁵.

As the cDNA clones under study were all identified with a monoclonal antibody, we examined whether the characterized

gene encodes the CHO1 antigen or another protein that carries the CHO1 epitope. Polyclonal antibodies were generated against bacterially expressed 70K and 50K C-terminal portions of the protein encoded by this cloned gene, and then affinity-purified against the 50K fragment which does not share any obvious similarity to other kinesin-like proteins. These antibodies stain mitotic PtK₁ cells in a pattern that completely overlaps that seen with CHO1 (Fig. 2). The protein undergoes a striking redistribution during the cell cycle; it moves from the nucleus to the spindle early in mitosis, concentrates at the interzone at anaphase and finally localizes to the midbody after cytokinesis⁴. The single difference seen between the two staining patterns is an affinity of the fusion protein antibodies for spindle poles through anaphase. This difference might arise from the different classes of antibodies involved (CHO1 is a monoclonal IgM, whereas the antibodies against fusion protein are polyclonal IgGs), or from cross-reactivity of the polyclonal antibodies with other kinesin-like proteins. Nonetheless, our immunolocalization data suggest that the cloned gene encodes a kinesin-like protein that associates specifically with the mitotic spindle; we propose to call it human mitotic kinesin-like protein-1 (MKLP-1).

To examine the binding of MKLP-1 to microtubules *in vitro*, we prepared kinesin and kinesin-related proteins from extracts of HeLa cells by microtubule affinity¹⁶ and analysed immunoblots of these preparations probed with CHO1 affinity-purified anti-MKLP-1 antibodies and a polyclonal anti-kinesin raised against the motor domain of the *Drosophila* kinesin heavy chain¹⁷. Staining with Coomassie blue (Fig. 3a) shows that the

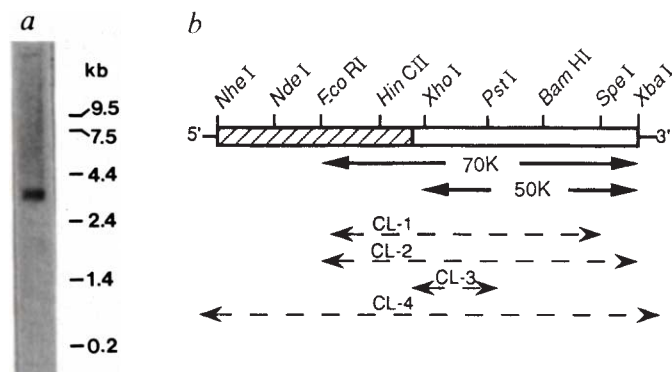


FIG. 1 a, Northern blot of HeLa cell total RNA probed with a 2.4 kb fragment of the gene encoding the CHO1 antigen. Molecular markers (kb) are indicated. b, Map of the gene encoding a CHO1 antigen. Unique restriction enzyme sites are marked. The region that contains the kinesin-like motor domain is hatched. A 70K C-terminal polypeptide used to elicit polyclonal antibodies, and a 50K C-terminal fragment used for immunization and to prepare an affinity column, are marked. The relative positions of four immunopositive clones (CL-1-4) are shown. c, Nucleotide sequence of a gene encoding a CHO1 antigen and its predicted amino-acid sequence (single-letter code). The context of the first in-frame methionine codon is a good match to the eukaryotic consensus for translation initiation²⁷. The ORF initiated at this methionine residue spans 2,913 bp. The first termination codon is followed by several in-frame stops and a polyadenylation sequence between bases 3,059 and 3,063 (overlined). Putative sites for a nuclear localization signal (residues 7-11), and a consensus phosphorylation site for p34^{cdc2} kinase (residues 13-20) are underlined. Regions shared by many kinesin-like proteins: the ATP-binding site (residues 113-117), the SSRSH domain (residues 296-300) and the LAGSE domain (residues 334-340) are boxed.

METHODS. Total HeLa cell RNA was isolated as described²⁸, and 10 µg was separated by electrophoresis on a 1% agarose/formaldehyde gel, blotted to 0.45 µm nitrocellulose (Schleicher and Schuell) and probed with a 2.4-kb fragment of the gene encoding the CHO1 antigen. An oligo(dT)-primed HeLa cell cDNA library in λ ZAP (Stratagene) was screened with the CHO1 antibody. Nested deletions of cDNA inserts were prepared by digestion of linearized plasmids with Exonuclease III (Promega). Both strands of overlapping inserts were sequenced using Sequenase 2.0 (USB). The sequence has been deposited in the EMBL/Genbank database under accession number X67155.

(-76: ttcgtgatggattcagactactcctcaaccactcttctaatgattggacaacaaagaaaaa
1: aaagaaaaaaagccatgttgcagcgagagctaaagacacccggaacccctcagctgaa
11: M L S A R A K T P R K P T V K
45: aaaaaggtcccaaacgaacgaacccatgttggaactctgaggtgagcagctgggctt
16: K G P K R T L K T Q L G Y C R V R L G F
105: tctgtatcaagagtggttgcataagagctcaataataacacagcttgcagctcactcc
36: P D Q E C C I E V I N N T T V Q L H T P
165: tgagggtcagacactcaaccgaatgaaggactcaaggagactcagattatcattaaaca
56: E G Y R L N R N G D Y K E T O Y S P R K
225: agtattggcactcaccacccaggaagactcttctgattgttggttaactctgtgt
76: V F G T H T T Q K E L D V V A N P L V
285: caatgacctcattcattggcaaaaatgttcttcttcttcttcttcttcttcttcttctt
96: N D L I H G K N G L F T Y O V T G S G
345: aaaaactcacacatgactgttctccagggaaggagggctgcttctctgttcttcttga
116: K T H T M T G S P G E G G L L P R C L D
405: catgatctttaaagctatagggtcatttcaagctaaacgatagttttcaaatcattga
136: M I P N S I O S F Q A K R Y V F K S N D
465: taggaatagtgatgatacagctgtgaggttgatgcttattagaacgtcagaaaagaga
156: R N S M D I Q C E V D A L L E R Q K R E
525: agctatgccaactcacaagactcttcttcttcttcttcttcttcttcttcttcttctt
176: A M P N P K T S S S K R Q V D P E F A D
585: tatgatactgtacagaattctgcagaagcagaaggttgatgaagatgctgtctatgt
196: M I T V Q E F C K A E E V D E D S V Y G
645: tggatttctcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttctt
216: V P V S Y I E I Y N N Y I Y D L L E E V
705: ggcgtcttccataaaacccaaactcccaactcaaatgtcttcttcttcttcttcttctt
236: P F D P I N P N L H N L N C P V K I K N
765: ccaatacatgtatgttgcaggatgtcagaagtgaaagtgaagtgaagtgaagtgaagtga
256: H N M Y V A G C T E A V G V K S T E B A F
825: tgaagtcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttctt
276: E V F W R O G K K R R I A N T H L N R E
885: gtcacagcgttccatcagctgttcttcttcttcttcttcttcttcttcttcttcttctt
296: S S R S H S V F N I K L V Q A P L D A D
945: tggagactatgttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttctt
316: G D N V L Q E K E Q I T I S Q L S L V D
1005: tcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttctt
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1065: ttttaataataactcagtcactaactgagctgaagaacatgtatgagtgcttcttcttctt
356: G N I N Q S L M T L R T C M D V L R E N
1125: ccaatgctatggaactcaacagatgttcttcttcttcttcttcttcttcttcttcttctt
376: Q M Y G T N K M V P Y R D S K L T H L F
1185: caagaactacttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttctt
396: K N Y P D G E G K V R M I V C V N P K A
1245: tgaagattatgaagaaacttgcagctcagctgatttcttcttcttcttcttcttcttcttctt
416: E D Y E E N L Q V M R F A E V T Q R V E
1305: agtagcaagcctgtagacagcaatgttcttcttcttcttcttcttcttcttcttcttctt
436: V A R P V D K A I C G L T P G R R Y R N
1365: ccagctcagagctcagcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttctt
456: Q P R G P V G N E P L V T D V L Q S F
1405: tccacttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttctt
476: P P L P S C E I L D I N D E Q T L P R L
1485: gattgaagccttagagaaacgacataactacgacacatgatgatgatgatgatgatgatgat
496: I E A L E K R H N L Q R M I D E F N K
1545: acaatcattgcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttctt
516: Q S N A F K A L L Q E F D N A V L S K E
1605: aaaccacatgcaagggaacataatgaaggaagagatgatctcagacagaaattgga
536: N H M Q G K L N E K E K M I S Q Q K L E
1665: aatgaacagcactggaagaaac
556: I E R L E K K N K T L E Y K I E I L E K
1725: acaactcattctatgaggaagataaacgcaatttgcacaggaacttgaactcagaa
576: T T T I Y E E D K R N L Q Q E L E T Q N
1785: ccagacactcagcagacagcttcttcttcttcttcttcttcttcttcttcttcttcttcttctt
596: Q K L Q R Q F S E K R R L E A R L Q G M
1845: ggtgacagaaacgacacatgaagtgaggagaaagatgtgagcgttagatgtgagcagcaaca
616: V T E T T M K W E K E C E R R V A A K Q
1905: gctgagatgcagaaataactctgtgttcttcttcttcttcttcttcttcttcttcttctt
636: L G M Q N K L W V K D E K L K Q L K A I
1965: tgttactgacactaaaactgagaagccagagacccctctcgggagcgagatcgagaaaa
656: V T E P K T E K K P E R P S R E R D R E K
2025: agtactcaagatctgttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttctt
676: V T Q R S V S P S P V P L L F Q P D Q N
2085: cgcacacttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttctt
696: A P P I R L R E R R S R S A G D R W V D
2145: tcatgaagccgctcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttctt
716: H K P A S N M Q T E T V M Q P H V P H A
2205: catcacagatctgttgcacatgaaggcactagctaaagtgtgagagatcatgctgac
736: I T V S V A N E K A L A C E K Y M L T
2265: ccaccaggaactagcctcagatgggagatgaaactaaactaaactaaactaaactaaactaaact
756: H Q E L A S D G E I E T K L I K G D I Y
2325: taaaacaaaggggtgttggaactctgtcagtttcttcttcttcttcttcttcttcttcttctt
776: K T R G G G Q S V Q F T D I E T L K Q G
2385: atcccaaatggtagtgcacacgaagatcttccagctagcagcctcccaacacagatgg
796: S P N G S R K R R S S T V A F A Q P D Q
2445: tgcagagctgtgaatggagcgtgtgtagaacaaggtgttcttcttcttcttcttcttcttcttctt
816: A E S E W T R C R N K V F C G C E M R A
2505: aggatccagctggacactgatcagcagcagcagcagcagcagcagcagcagcagcagcagcagc
836: G S Q L D L I A T A S R G N P S A K S H E
2565: aactgacagctccagcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttctt
856: T D S P S T E R T F S F V W M I S R K P
2625: atgcaggaagcagcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttctt
876: C Q K Q S R S S C R T P A L V E N H G
2685: acctcagctacatcatcactgaccagaaataaagcttccctatggttccaaagacacac
896: P Q L E H T L T Q N K A F P M V P K T T
2745: tagtattcaacaaactgtatgtatgttcttcttcttcttcttcttcttcttcttcttcttctt
916: S I Q Q T L Y S V C F A I F N I N S R G
2805: aagactccttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttctt
936: R L L F S S L Y E F F I M F L K Y I S
2865: atgtatcattataaactaatcacaagctgttcttcttcttcttcttcttcttcttcttcttctt
956: C I L I N G
2925: tctagatctgtctgattttcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttctt
2985: tttctataacaaatgtcttattattagctgcaagatgcaactttagaactatttgaca
3045: attcagactttcaaaatgaagatgaaatgactggccaataaaccatttaggaaggt
3105: gttttgattctgtatattatcttcttcttcttcttcttcttcttcttcttcttcttcttcttcttctt
3165: aatcaaaagcactaagg.

principal proteins associated with microtubules under these conditions are a high-molecular-mass polypeptide, probably cytoplasmic dynein¹⁸ and a 115K polypeptide that binds strongly to antibodies against kinesin (Fig. 3b); this probably represents the heavy chain of HeLa kinesin. Figure 3c and d compare the staining of the same immunoblot probed with CHO1 and affinity-purified anti-MKLP-1. Despite the low abundance of MKLP-1 in these preparations, the main component stained on immunoblots is a band of M_r 100K. The extraction behaviours of the 100K antigens recognized by CHO1 and by anti-MKLP-1 are indistinguishable and, in addition, both CHO1 and anti-MKLP-1 antibodies immunoprecipitate a cross-reactive polypeptide of M_r 100K (data not shown). As all of the immunolocalization and immunochemical properties that define the CHO1 antigen are shared by human MKLP-1, we conclude that they are the same protein.

The microtubule affinities of MKLP-1 and HeLa kinesin are similar but distinguishable (Fig. 3b–d). Both proteins are extracted from microtubules by 100 mM NaCl and 2.5 mM Mg-ATP (lanes 13 and 14) and not by 100 mM NaCl and 2.5 mM AMP-PNP (5'-adenylylimidodiphosphate, an inactive ATP analogue; lanes 15 and 16). But MKLP-1 is not extracted by Mg-ATP without added NaCl, whereas kinesin is extracted (lanes 11 and 12). In the absence of Mg-ATP, MKLP-1 is partially extracted with 200 mM NaCl (lanes 7 and 8) and completely extracted by 300 mM NaCl (lanes 9 and 10), while kinesin is not. About 50% of the kinesin is extracted by 10 mM Mg-GTP, whereas MKLP-1 is not (lanes 17 and 18). These results confirm that MKLP-1 is kinesin-like, but show that it is distinct from kinesin.

To understand how the microtubule-binding of MKLP-1 might relate to its mitotic function, we studied the interaction in other *in vitro* assays. MKLP-1, expressed in bacteria, did not

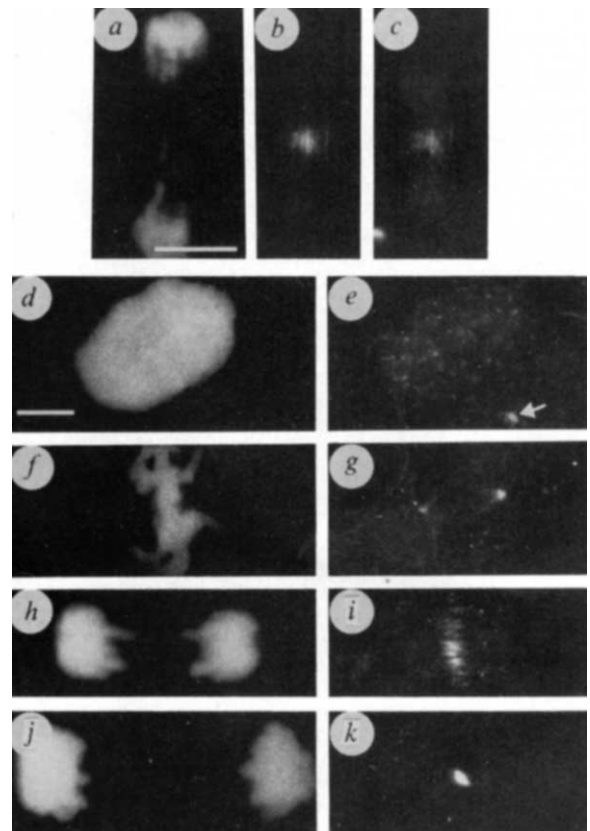
promote microtubules to glide over glass in conditions that yield motility of other microtubule motors (MKLP-1 adsorbed either directly onto glass or onto protein-coated glass). But we did observe ATP-sensitive bundling of microtubules by MKLP-1. We investigated the polarity of these microtubule bundles using microtubules grown from *Tetrahymena* pellicles¹⁹, in which the microtubule plus-ends radiate distally and the minus-ends are anchored²⁰. When bacterial extracts containing MKLP-1 are added, antiparallel microtubules form bundles between closely spaced pellicles (Fig. 4a–d, j–k). On addition of 15 mM Mg-ATP, antiparallel microtubules become unbundled (Fig. 4e, f, k and l) and eventually disperse individually. Substitution of ATP with AMP-PNP did not disperse the antiparallel bundles, but augmented them. Control preparations using bacterial extracts lacking MKLP-1 did not cause antiparallel microtubules to bundle (data not shown).

During the course of characterizing the cross-bridges, we observed that a few *Tetrahymena* axonemes remaining in the pellicle preparation moved towards the plus end of pellicle-initiated microtubules (Fig. 4g–i). To determine the polarity of the sliding microtubules, we supplemented the assay with *Chlamydomonas* axonemes that characteristically splay microtubules grown from their plus ends. MKLP-1 does not affect this splaying, so the axonemal plus-ends are obvious in these assays. When Mg-ATP is added, these axonemes moved towards the plus end of the pellicle-initiated microtubules with the axonemal plus-end trailing (Fig. 4m–o). These axonemes moved away from the pellicles at a constant rate of $4.0 \mu\text{m min}^{-1}$ (s.d. = 1.6, $n = 13$).

The mitotic localization of MKLP-1, and the observations that it bundles antiparallel microtubules *in vitro* and moves these polymers over one another at rates comparable to, although

FIG. 2 PtK₁ cells stained with affinity-purified anti-MKLP-1, CHO1 antibody and DAPI. a–c, The same cell in anaphase, stained with DAPI (a), CHO1 IgM (b) or anti-MKLP-1 (c). The patterns obtained with the two antibodies are superimposable. d, e, Interphase cell. Staining with antibodies raised against the C terminus of recombinant MKLP-1 reveals that MKLP-1 localizes to punctuate spots within the nucleus and also to the interphase centrosome (arrow). f, g, Metaphase cell. MKLP-1 stains the spindle poles intensely, and also faintly localizes to spindle fibers at this stage. h, i, Anaphase cell. MKLP-1 localizes to the spindle interzone in a pattern coincident with microtubules in this region (as indicated by double-staining for microtubules, data not shown). j, k, Late telophase cell. MKLP-1 localizes exclusively to the midbody. Scale bars, 5 μm .

METHODS. PtK₁ cells were grown on glass coverslips, lysed for 1 min at 23 °C in a microtubule-stabilizing buffer (PMEEG buffer: 100 mM PIPES, 5 mM MgSO₄, 5 mM EGTA, 0.5 mM EDTA, 0.9 M glycerol, pH 6.9) plus 0.5% Triton X100, then fixed by immersion in methanol for 7 min and acetone for 2 min at –20 °C. Fixed cells were rinsed in PBS (136 mM NaCl, 80 mM Na₂HPO₄, 3 mM KCl, 2 mM MgCl₂ and 1 mM KPO₄) containing 0.05% Tween-20 (PBST) and stained with primary antibodies for several hours at 37 °C. CHO1 IgM³ was used at 20 $\mu\text{g ml}^{-1}$ in PBST. Affinity-purified anti-MKLP-1 antibodies were prepared as follows. Fragments of MKLP-1 (2.4 and 1.3 kb) encoding 70K and 50K C-terminal fragments of MKLP-1 (see Fig. 1b) were subcloned into the expression vector pET5b (ref. 29). Recombinant protein expression was induced by adding 0.1 mM isopropyl β -D-thiogalactopyranoside (IPTG) to logarithmically growing cultures. Inclusion bodies were isolated as described³⁰, separated by SDS-PAGE and electroblotted to 0.2- μm nitrocellulose. Strips containing these proteins were dissolved in 250 μl of dimethylsulphoxide mixed with complete Freund's adjuvant (Sigma) and used to immunize four New Zealand white rabbits (Hazelton laboratories). Three subsequent boosts used protein mixed with incomplete Freund's adjuvant. MKLP-1-specific antibodies were purified from the immune sera by affinity chromatography on a column of CNBr-sepharose (Sigma) to which was conjugated the 50K C-terminal fragment of MKLP-1 (see Fig. 1b). Antibodies that did not bind to the column were collected and used for control staining at 50 $\mu\text{g ml}^{-1}$ in PBST. Bound antibodies were eluted with 100 mM glycine, pH 2.4, and used for immunostaining at 25 $\mu\text{g ml}^{-1}$ in PBST. Secondary antibodies, Texas red-donkey anti-rabbit (Jackson Immunochemicals) and FITC-goat anti-mouse (US Biochemicals), were diluted to 10 $\mu\text{g ml}^{-1}$ in PBST and applied to cells for 1 h at 37 °C. After antibody staining, cells were



washed in PBST, stained for 1 min in 0.05% DAPI (diaminophenylindole). Images were taken directly from a Zeiss Universal microscope and recorded on T-MAX p3200 film (Kodak).

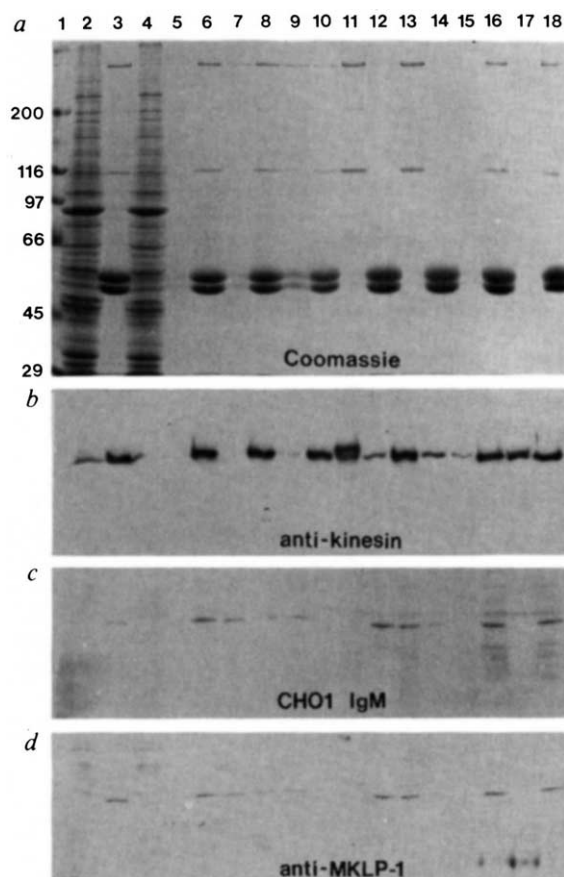
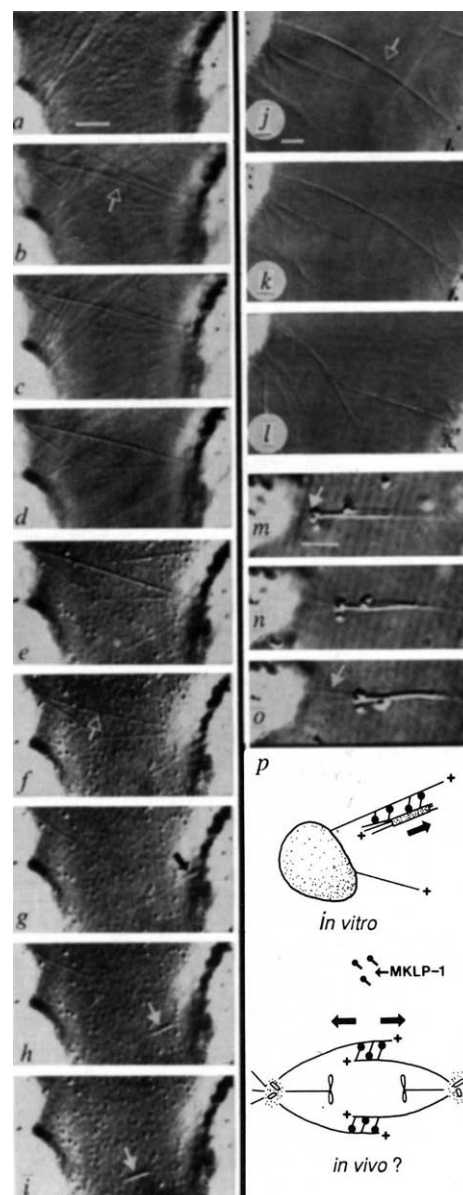


FIG. 3 Immunoblot analysis of the microtubule binding of MKLP-1 and the HeLa kinesin heavy chain. HeLa cells were sonicated, depleted of endogenous microtubules and then clarified by centrifugation to prepare a high-speed supernatant. This preparation was supplemented with taxol-stabilized microtubules, polymerized from purified bovine brain tubulin³¹, to prepare a microtubule pellet which was then extracted with various combinations of NaCl and/or nucleotides. *a*, Coomassie blue-stained 7.5% acrylamide gel of microtubule affinity fractions. Lane 1, Molecular mass markers (K); 2, HSS; 3, MTP; 4, high-speed supernatant depleted of microtubules; 5, 100 mM NaCl extract; 6, pellet from 5; 7, 200 mM NaCl extract; 8, pellet from 7; 9, 300 mM NaCl extract; 10, pellet from 9; 11, 0 mM NaCl, 2.5 mM Mg-ATP extract; 12, pellet from 11; 13, 100 mM NaCl, 2.5 mM Mg-ATP extract; 14, pellet from 13; 15, 100 mM NaCl, 2.5 mM Mg-AMP-PNP extract; 16, pellet from 15; 17, 100 mM NaCl, 10 mM Mg-GTP extract; 18, pellet from 17. *b*, Immunoblot corresponding to the gel in *a*, probed with antibodies against *Drosophila* kinesin motor domain. *c*, Immunoblot corresponding to the gel in *a*, probed with CHO1 IgM. *d*, The same immunoblot as in *c*, stripped and reprobed with affinity-purified anti-MKLP-1 antibodies. The migration of HeLa kinesin heavy chain and MKLP-1 are indicated with single and double dots, respectively. In these preparations the molar ratio of HeLa kinesin to MKLP-1 is at least 100 to 1.

METHODS. HeLa kinesin and kinesin-related proteins were prepared from cells grown in suspension culture as described¹⁶. Microtubule pellets were washed, and resuspended in various combinations of PMEG plus NaCl and/or nucleotide, incubated at 23 °C for 30 min at 37 °C for 10 min then centrifuged at 50,000*g* for 30 min at 23 °C. The resulting supernatants were collected and the pellets were resuspended to form their original volumes in PMEG. Fractions were separated by SDS-PAGE and electroblotted to 0.45- μ m Immobilon polyvinylidene fluoride membranes (Millipore). Immunoblots were blocked in PBST + 1% gelatin for 2 h at 23 °C and probed with primary and secondary antibodies diluted in PBST + 0.5% gelatin for 1 h each at 23 °C. Signals produced by horseradish peroxidase-labelled secondary antibodies (Sigma) were detected using enhanced chemiluminescence (Amersham) and recorded on Kodak X-OMAT film. To reprobe blots, membranes were stripped with 10 mM β -mercaptoethanol and 0.5% SDS in 50 mM Tris, pH 6.7, 52 °C for 30 min then rinsed in PBST.

FIG. 4 MKLP-1 cross-bridges antiparallel microtubules and translocates axonemes along microtubules. *a*, Two closely spaced *Tetrahymena* pellicles have grown dense arrays of microtubules before the addition of MKLP-1. *b*, 20 s after the addition of MKLP-1, some microtubules from both pellicles begin to interact (arrow). *c*, 47 s after MKLP-1 was added. The bundling of antiparallel microtubules is more prominent, as a 'bridge' is formed between the two pellicles. *d*, When the tubulin concentration is lowered, the most stable microtubules observed are those that emanate from both pellicles and form bridges. More microtubule bridges can be seen in other focal planes (not shown). *e*, 3 min after 15 mM Mg-ATP is added, the microtubules that form this bridge begin to disperse. Also note the microtubule below the bridge; it serves as the 'track' for axoneme movement in *g-i*, below. The end of this microtubule is marked with an asterisk. *f*, 5.5 min after the addition of Mg-ATP, the microtubule bridge is thinning further (arrow). *g*, After the bridge has completely dispersed, an axoneme bound to the base of a microtubule track (arrow) begins moving out away from the pellicle. *h*, 30 s after it began moving, the axoneme (arrow) has moved 6 μ m. *i*, 70 s after it started to move, the axoneme (arrow) reaches the end of its microtubule track and stops, having travelled a total of 11 μ m. *j*, Two different pellicles with a bridge forming 40 s after the addition of MKLP-1 (arrow). *k*, The same bridge 30 s later, but before the addition of 15 mM Mg-ATP. *l*, Six min after the addition of Mg-ATP, the microtubules in the bridge begin to splay apart. *m*, *Chlamydomonas* axoneme bound to pellicle-initiated microtubules before Mg-ATP addition. (Arrow denotes the axonemal plus-end.) *n*, The same axoneme 30 s after adding Mg-ATP. *o*, The same axoneme



Autoimmune diabetes as a consequence of locally produced interleukin-2

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DURING cell differentiation in the thymus, self-reactive T cells can be generated. The majority of these seem to be deleted after intrathymic encounter with the relevant autoantigen¹. As all self antigens are unlikely to be present in the thymus, some autoreactive T cells may escape censorship. Here we study the fate of these cells using transgenic mice expressing the class I molecule H-2K^b (K^b) in the insulin-producing β -cells of the pancreas^{2,3}. These mice were crossed with mice transgenic for genes encoding a K^b-specific T-cell antigen receptor (TCR)⁴ which could be detected using a clonotype-specific monoclonal antibody⁵. Although T cells expressing the highest level of transgenic TCR were deleted intrathymically in double-transgenic mice, K^b-specific T cells were detected in the periphery. These cells caused the rejection of K^b-expressing skin grafts, but ignored islet K^b antigens even after priming. But when double-transgenic mice were crossed with transgenic mice expressing the lymphokine interleukin-2 in the pancreatic β -cells⁶, there was a rapid onset of diabetes. These results indicate that autoreactive T cells that ignore self antigens may cause autoimmune diabetes when provided with exogenous 'help' in the form of interleukin-2.

We have previously shown that mice expressing the allogeneic class I protein, H-K^b, in islet β -cells (RIP-K^b mice) did not develop autoimmunity, although a completely nonimmune form of diabetes ensued as a result of the overexpression of transgene product². These mice were tolerant of K^b-bearing skin, but tolerance waned with age, although the islets remained free from infiltration. At that time, we interpreted these results in favour of the notion of peripheral tolerance, and suggested that the loss of tolerance might be related to the loss of some β -cells and hence to a lower concentration of K^b (ref. 7). Our recent work, however, suggests that the tolerance to K^b-bearing skin is in fact due to the intrathymic expression of a few molecules of transgenic K^b, which was only detectable by the highly sensitive polymerase chain reaction⁸. This was sufficient to induce K^b-specific tolerance in normal T cells maturing through a transplanted thymus taken from RIP-K^b mice (Fig. 1a, b). It was necessary to determine whether so few molecules expressed intrathymically could delete all K^b-specific T cells or only high-affinity T cells and, if the latter were the case, what the fate could be of the low-affinity cells that escaped thymus censorship.

We therefore produced double-transgenic mice by mating RIP-K^b mice to mice transgenic for a K^b-specific TCR that could be detected by the monoclonal antibody Des (Des-TCR mice). Flow cytometric analysis of lymph node cells from Des-TCR $\times$ RIP-K^b mice revealed the presence of cells positive for the K^b-specific clonotype and CD8 (Des⁺CD8⁺ cells) (Fig. 2a, b). It was notable, however, that the number of cells expressing the highest density of TCR was greatly reduced compared with cells in single transgenic mice (Fig. 2c). Further analysis showed a 66% reduction (5.4 to 1.8%) in the proportion CD4⁺CD8⁺ cells in the thymus of double- compared with single-transgenic mice

seen 67 s after adding Mg-ATP. Note that the characteristically splayed plus-end of the axoneme is facing towards the pellicle. *p*, Diagram suggesting how the antiparallel sliding of axonemes observed along pellicle-initiated microtubules *in vitro* (top) might relate to spindle elongation *in vivo* during anaphase B (bottom). Scale bars, 5 μ m.

METHODS. A 3.0-kb *NheI*-*XbaI* restriction fragment containing the complete coding sequence of MKLP-1 was subcloned into pET5b as described. Protein expression was induced with 0.1 mM IPTG for 10 h at room temp. Soluble MKLP-1 was prepared as described³². *Tetrahymena* pellicles and other assay components were prepared as described¹⁹. *Chlamydomonas* axonemes were prepared according to ref. 33. Microtubules were grown from pellicles as described¹⁹. MKLP-1 was then added in the presence of 25 μ M tubulin with or without axonemes and allowed to incubate for 5 min. Mg-ATP (15 mM) was then added with 10 μ M tubulin (a concentration that does not promote microtubule polymerization in our assay) and sequences were videotaped. Control extracts were prepared exactly as described above from cells carrying the plasmid pET5b that did not contain the MKLP-1 cDNA.

slightly faster than, spindle elongation *in vivo*²¹, imply that this enzyme may provide the force for anaphase B (Fig. 4p). Such mechano-chemical activity has long been suspected to be involved in spindle elongation (reviewed in ref. 22).

But MKLP-1 may not be the only kinesin-like protein with a role in mitosis. For example, the gene encoding another human mitotic protein, CENP-E²³, encodes a unique, kinesin-like protein that is distinct from MKLP-1 (T. Yen, personal communication). Thus mammalian spindles, like those of yeast^{24,25}, contain at least two (and probably several) kinesin-like motor enzymes. Like other kinesin-like proteins²⁴⁻²⁶, MKLP-1 may engage in several mitotic functions, some of which could be shared with other kinesin-like proteins. Our observation that antibodies against MKLP-1 inhibit mitotic progression⁴, combined with the finding that this protein can bundle and cause sliding of antiparallel microtubules, indicates that MKLP-1 may play a key role in spindle elongation during anaphase B. □

Received 10 July; accepted 21 August 1992.

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ACKNOWLEDGEMENTS. C.N. dedicates this paper to F. Nislow. We thank V. I. Gelfand and E. Vaisberg for the anti-kinesin antiserum and M. Suffness for taxol; M. Koonce and M. Perry for technical suggestions; S. Dutcher and S. King for *Chlamydomonas* advice; T. Yen shared data before publication and L. Pillus made helpful suggestions on the manuscript. This work was supported by grants from the NIH and NIGMS (V.A.L.).

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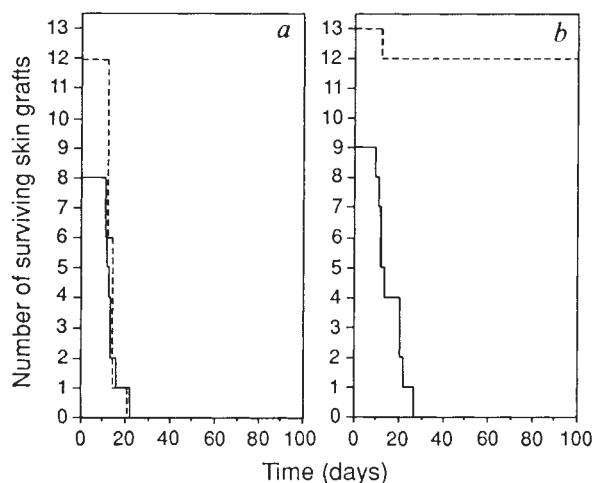


FIG. 1 Rejection of third party bm11 (a) or K^b-bearing C57BL/6 (b) skin grafts by T cells maturing through a bm1 RIP-K^b (dashed line) or littermate (full line) thymus graft.

METHODS. Six-week-old bm1 mice were thymectomized and, after two weeks, were irradiated (1,000R) and reconstituted with bm1 bone marrow. These mice were also injected with 0.1 ml Thy-1-specific ascites fluid (T24; ref 14) to deplete radiation-resistant T cells. T-cell-deficient mice were then grafted under the kidney capsule with either a neonatal bm1 RIP-K^b or littermate thymus. After a further 8 weeks to allow T cells to reconstitute, mice were grafted¹² with tail skin from bm11 and C57BL/6 mice and the graft survival was assessed.

(Fig. 2d, e). This confirms that a few molecules of K^b must have been present intrathymically, mediating the deletion of T cells with the highest density of TCR and presumably with the highest avidity for K^b (Fig. 2f).

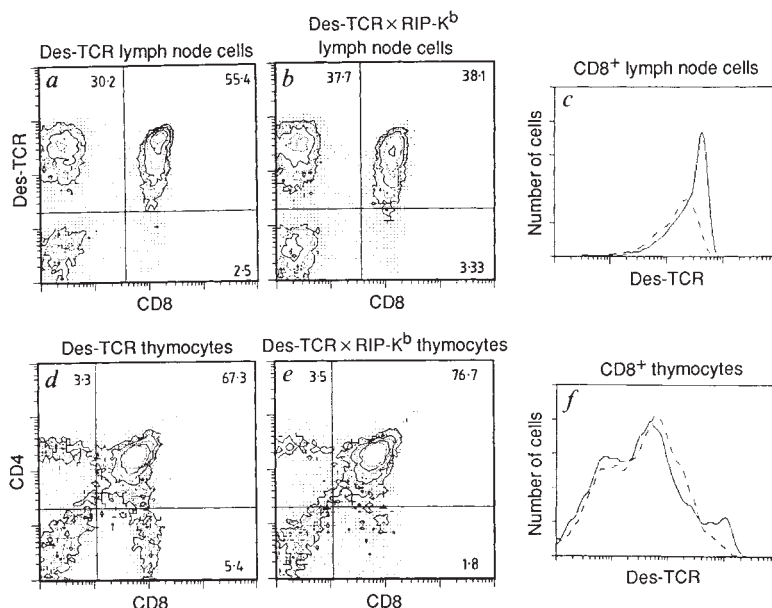
To determine whether CD8⁺ cells expressing the lower density of TCR in Des-TCR × RIP-K^b mice were immunocompetent, young double- and control single-transgenic mice were grafted with K^b-bearing skin. These grafts were rejected by both sets of mice (Fig. 3a, b). To reconcile this with our previous finding that young (not more than 12 weeks old) RIP-K^b mice were tolerant of K^b-bearing skin⁸, we suggest that 'lower avidity'

K^b-specific cells escape thymic deletion in RIP-K^b mice and must reach a certain threshold number before they can reject skin grafts. The time required to reach this threshold must be more than 12 weeks in RIP-K^b mice, but would obviously be reduced in Des-TCR × RIP-K^b mice, which essentially generate only K^b-specific T cells. The immunocompetence of T cells in double-transgenic mice suggests that K^b-specific T cells are unaffected by β-cell K^b antigens. This supports the view that antigens expressed on β-cells are ignored, and is in accordance with studies using transgenic mice expressing the lymphocytic choriomeningitis virus (LCMV) glycoprotein or nucleoprotein in the β-cells^{9,10}. Such mice were neither immune nor tolerant, but after infection with LCMV, CD8⁺ T cells were activated and produced selective β-cell damage and diabetes. In our Des-TCR × RIP-K^b mice, however, islet infiltration has only been observed in 2 out of 17 mice primed by skin grafts (data not shown). Our current interpretation of this result is that K^b-specific T cells that escaped thymus censorship in Des-TCR × RIP-K^b mice are of insufficient avidity to cause a destructive response in the islets, even after cross-stimulation by skin-graft antigens.

As interleukin-2 (IL-2) enhances the *in vitro* response of K^b-specific cells from RIP-K^b mice³, we wondered whether local production of IL-2 might activate K^b-specific cells in double-transgenic Des-TCR × RIP-K^b mice. To this end, we generated transgenic mice that expressed IL-2 in the β-cells⁶ (RIP-IL-2 mice). We had already shown that IL-2 production in these mice attracted mononuclear cells around the islets. Triple transgenic mice, produced by mating the Des-TCR × RIP-K^b mice to homozygous RIP-IL-2 mice, developed severe diabetes as early as one week after birth (Fig. 4), and most mice died when they were between 2 and 10 weeks old. Diabetes was not observed by day 40 either in RIP-IL-2 or in Des-TCR × RIP-IL-2 mice. The severe form of diabetes observed in triple-transgenic mice indicates that K^b-specific T cells can destroy β-cells, provided these cells express both IL-2 and K^b. This is in contrast to the findings we obtained previously in RIP-K^b × RIP-IL-2 double transgenic mice, which did not show immune-mediated destruction of β-cells but developed diabetes at about 4 weeks of age from the nonimmune effects of overexpression of K^b molecules⁶. As the onset and extent of the disease was identical to that of the RIP-K^b mice, any autoimmune component developing after

FIG. 2 Flow cytometric analysis of lymph node cells and thymocytes from H-2^k single-transgenic Des-TCR and double-transgenic Des-TCR × RIP-K^b mice. Lymph node cells prepared from adult Des-TCR (a) or Des-TCR × RIP-K^b (b) mice were analysed for expression of the Des⁺ TCR and CD8 molecules. Des⁺CD8⁺ cells shown in Fig. 1a and b are CD4⁺ cells and do not respond to K^b-expressing stimulator cells *in vitro* (L. Kjer-Nieslen, unpublished data). Profiles of Des-TCR (full line) and Des-TCR × RIP-K^b (dashed line) lymph node cells were then gated to include CD8⁺ cells only, and these cells were analysed for expression of the Des⁺ TCR (c). Thymocytes prepared from 6-week-old Des-TCR (d) and Des-TCR × RIP-K^b (e) mice were analysed for the expression of CD4 and CD8 molecules. Des-TCR and Des-TCR × RIP-K^b thymocytes were also stained for the Des⁺ TCR and CD8, and then gated on CD8⁺ cells to test for TCR expression (f).

METHODS. Single-cell suspensions of lymph node cells and thymocytes were stained for 30 min at 4 °C with fluorescein isothiocyanate (FITC)-conjugated CD8-specific monoclonal antibody (Becton Dickinson) and either biotin-conjugated CD4-specific monoclonal antibody (Becton Dickinson) or biotin-conjugated Des monoclonal antibody specific for the idiotype of the transgenic TCR⁵. Cells were washed twice and incubated for a further 30 min with phycoerythrin-conjugated streptavidin (Caltag Laboratories). After washing, samples were analysed by two-colour flow cytometry in a FACScan flow cytometer (Becton Dickinson) with 10,000 events collected. The intensity of fluorescence is shown in arbitrary units on a four-decade



logarithmic scale. Histograms were produced by gating out cells that did not stain with the CD8-specific antibody.

significant accumulation of low-avidity K^b -specific T cells would have been masked by this nonimmune diabetes. In the triple-transgenic mice, in which the frequency of K^b -specific T cells is very high, the onset of autoimmunity would of course be accelerated.

As the pancreata of RIP-IL-2 transgenic mice have abundant leucocytic infiltrates⁶, the infiltrating cells could be analysed by flow cytometry (data not shown). Infiltrates of the 14-day-old triple-transgenic mice contained a large proportion of Des^+CD8^+ T cells (13, 17 and 18% in three mice tested), many of which expressed the IL-2 receptor. By contrast, age-matched Des -TCR $\times$ RIP-IL-2 mice had very few Des^+CD8^+ cells (0–4.7%), presumably because of the absence of K^b stimulation. Some of these cells, however, expressed the IL-2 receptor, suggesting that encounter with K^b was not obligatory for the induction of this receptor.

These experiments yield two important results. First, there was no evidence for clonal inactivation: immunocompetent K^b -specific T cells were present in the periphery of transgenic mice expressing K^b on β -cells and were neither tolerized nor immunized by these class I molecules. Although these cells rejected K^b -bearing skin specifically, such priming did not allow infiltration of islet tissue. Second, local production of IL-2 can lead to the stimulation of an antigen-specific autoimmune response.

The lack of peripheral tolerance in our present model is in contrast to other reports using extrathymic K^b or haemagglutinin expression^{4,11–13}. This may be due to differences in the site of K^b expression altering the toleragenic outcome^{4,11}. It is also difficult to rule out thymic deletion without the ability to detect TCR idiotypes specific for the particular antigen^{12,13}. Perhaps this explains why, in earlier studies using different TCR transgenic mice (F3) we did not detect deletion of cells expressing high levels of K^b -specific TCR in F3 $\times$ RIP- K^b mice¹². A notable

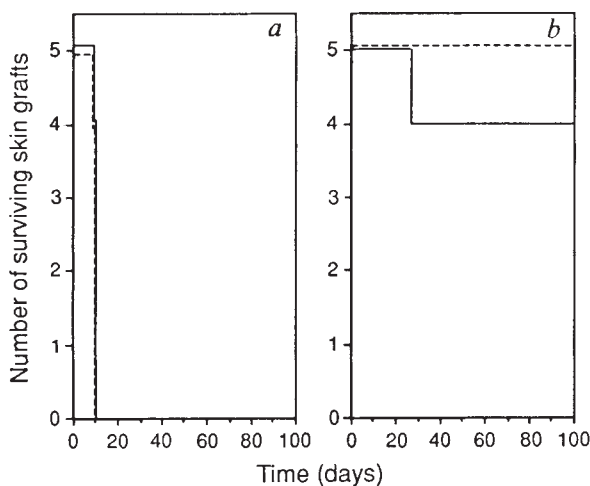


FIG. 3 Rejection of K^b -bearing skin grafts (a) or littermate grafts (b) by single-transgenic Des-TCR (full line) and double-transgenic Des-TCR $\times$ RIP- K^b (dashed line) mice.

METHODS. Six-week-old H-2^k Des-TCR and Des-TCR $\times$ RIP- K^b mice were grafted with tail skin from B10.BR mice transgenic for the natural K^b gene (M. W. Hoffmann, unpublished) or with tail skin from littermate controls. The latter transgenic mice (BR- K^b mice) were derived from (B10.BR $\times$ CBA)F₂ founder mice that were backcrossed to B10.BR for 5 generations, resulting in the occasional late rejection of littermate skin owing to minor histocompatibility differences (as seen for one Des-TCR mouse in b). Similar results were obtained when young Des-TCR $\times$ RIP- K^b mice were thymectomized and skin-grafted 6 weeks later (data not shown). This argues against the possibility that newly derived Des^+CD8^+ T cells, which had not yet encountered islet K^b antigens, were responsible for the lack of tolerance in double-transgenic mice.

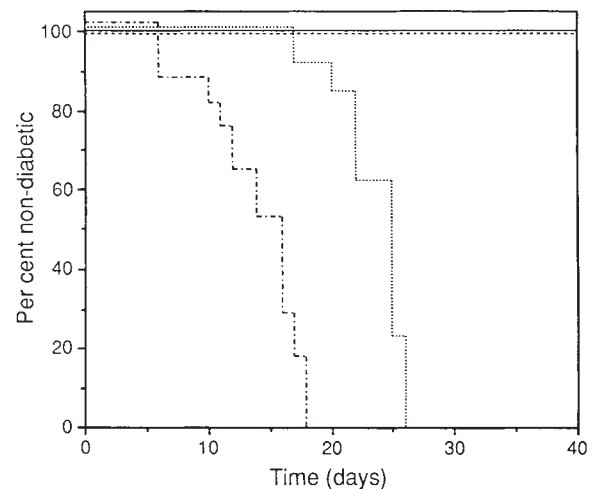


FIG. 4 Diabetes incidence in triple-transgenic Des-TCR $\times$ RIP- K^b $\times$ RIP-IL-2 mice (---) and their littermates, Des-TCR $\times$ RIP-IL-2 (----), RIP- K^b $\times$ RIP-IL-2 (.....), and RIP-IL-2 (—).

METHODS. Des-TCR $\times$ RIP- K^b mice were mated to mice homozygous for the RIP-IL-2 transgene and offspring were tested for glucosuria twice weekly using Diastix (Bayer Diagnostics).

difference between these mice and Des-TCR $\times$ RIP- K^b double transgenic mice is that the former were tolerant of K^b -bearing skin. This may partly relate to differences in the peptides recognized by the two receptors or to age differences of the mice examined: the former were 4–5 weeks old, the latter 6 weeks old. Perhaps insufficient low-avidity cells had accumulated by 4–5 weeks.

It has been suggested that autoimmune disease may arise by the crossreactive stimulation of self-reactive T cells on an environmental antigen. Failure of the K^b -specific T cells in Des-TCR $\times$ RIP- K^b mice to cause islet damage, even after skin graft priming, suggests that this process critically depends on the avidity of self-reactive T cells. The rapid onset of disease in triple transgenic mice suggests that 'low-avidity' cells, present in large numbers, can produce a destructive response if sufficient help is provided in the form of IL-2. This raises the question of why IL-2 released by T cells in the normal process of fighting infections does not normally cause autoimmune disease. This may relate to the very low frequency of autoreactive T cells in a normal repertoire and to the relatively short duration of IL-2 production during most infections. Chronic infections that stimulate a long-term low level of immunity may, however, provide a more stable source of IL-2 for the activation of cells with the potential to cause autoimmune disease. □

Received 26 May; accepted 24 August 1992.

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ACKNOWLEDGEMENTS. We thank J. Donohue, K. Holmberg, L. Oxbrow, A. Simos and J. Falso for technical assistance. This work was supported by grants from the NIH and the National Health and Medical Research Council of Australia. W.R.H. is supported by a fellowship from the Cancer Research Institute (USA).

Long-term proliferation of mouse primordial germ cells in culture

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PRIMORDIAL germ cells (PGCs) are first identifiable as a population of about eight alkaline phosphatase-positive cells in the 7.0 days postcoitum mouse embryo¹. During the next 6 days of development they proliferate to give rise to the 25,000 cells that will establish the meiotic population². Steel factor is required for PGC survival both *in vivo*³ and *in vitro*^{4,5} and together with leukaemia inhibitory factor stimulates PGC proliferation *in vitro*⁶. In feeder-dependent culture, PGCs will proliferate for up to 7 days, but their numbers eventually decline and their proliferative capacity is only a fraction of that seen *in vivo*^{6,7}. Here we report a further factor that stimulates PGC proliferation *in vitro*, basic fibroblast growth factor (bFGF). Furthermore, bFGF, in the presence of steel factor and leukaemia inhibitory factor, stimulates long-term proliferation of PGCs, leading to the derivation of large colonies of cells. These embryonic germ cells resemble embryonic stem cells, pluripotent cells derived from preimplantation embryos, or feeder-dependent embryonal carcinoma cells, pluripotent stem cells of PGC-derived tumours (teratomas and teratocarcinomas)⁸. To our knowledge, these results provide the first system for long-term culture of PGCs.

We have previously shown that the transmembrane form of steel factor is required for PGC survival in culture and we have observed that a soluble factor produced by the mouse embryonic fibroblast STO cell line can stimulate proliferation⁴. Hogan and colleagues have shown that this factor is probably leukaemia inhibitory factor (LIF)⁶. LIF exists as a diffusible form or as a matrix-associated form⁹. Although STO cells produce LIF^{9,10}, addition of recombinant murine LIF to the culture medium enhances PGC proliferation on STO cells in a dose-dependent manner, with a peak response at around 1,000 units ml⁻¹ (Fig. 1a). Because of the ability of bFGF to stimulate both diffusible and matrix-associated LIF production by feeder cells¹¹, we also analysed the effect of bFGF on PGC proliferation. The bFGF stimulates dose-dependent proliferation of PGCs with a peak response at around 1 ng ml⁻¹ (Fig. 1b). At their optimal concentrations, LIF and bFGF stimulate PGC proliferation but PGC numbers decline after 5 days (data not shown). But we observe continued growth of some PGCs in the presence of LIF and bFGF, leading to the formation of increasingly large PGC colonies (Fig. 2). Initially, PGCs are identifiable in culture as single alkaline phosphatase-positive cells (Fig. 2a), which eventually form small colonies either by aggregation or clonal division (Fig. 2b, c). These colonies initially enlarge as a cellular monolayer (Fig. 2c, d) but eventually form multilayered clumps growing on top of the STO cell feeder layer (Figs 2e, f and 3c). In the presence of bFGF and LIF, multilayered colonies of PGCs were easily identifiable 9 days after initial plating onto STO feeder layers. In the absence of bFGF and LIF, multilayered colonies of PGCs have not been observed (refs 4–7, and our unpublished observations). We estimate that between 8–23% of plated PGCs go on to form multilayered colonies (data not shown). Furthermore, we observe formation of these colonies from PGCs isolated from 8.5-days postcoitum (d.p.c.) embryonic fragments (Figs 1, 2 and 3) or from 11.5-d.p.c. genital ridges (data not shown). The large, multilayered colonies have been isolated and serially passaged and retain characteristics of PGCs. In the embryo, PGCs demonstrate alkaline phosphatase activity from 7.0 to 15.5 d.p.c.^{1,2} and express the carbohydrate differenti-

ation antigen stage-specific embryonic antigen-1 (SSEA-1) from 8.5 to 14.5 d.p.c.^{7,13}. Alkaline phosphatase and SSEA-1 are also definitive markers of PGCs in culture⁷. Like PGCs, the cells described here demonstrate alkaline phosphatase activity (Fig. 2) and express the SSEA-1 antigen (Fig. 3a, b). Pluripotent embryonic stem and embryonal carcinoma cells also express these markers¹⁴ and, interestingly, the large multilayered colonies of PGCs closely resemble these cells grown on feeder layers (Fig. 3c). These similarities are intriguing and we are currently testing the ability of these cells to differentiate *in vitro*, to form chimaeras and to form teratomas in histocompatible hosts. We propose calling these cells embryonic germ cells or EG cells.

A major problem in our understanding of the development of the mammalian germ line has been the inability to culture PGCs for significant periods of time. The failure of PGCs to proliferate *in vitro* at the same rate as they do *in vivo* suggests the requirement for factors other than just steel factor and LIF⁶. Here we demonstrate the first example of long-term proliferation of PGCs and provide a potential role for bFGF in regulating PGC proliferation. The bFGF may act through its ability to stimulate LIF production by the feeder cells, or alternatively it may bind directly to PGCs through an FGF receptor. It seems likely that EG cell derivation requires bFGF and LIF, rather than representing spontaneous (growth factor-independent)

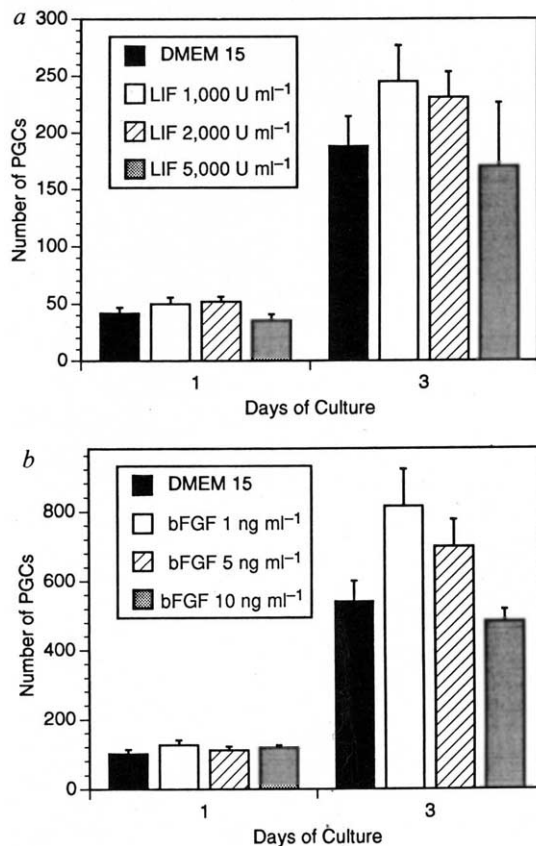


FIG. 1 Analysis of 8.5-d.p.c. PGC proliferation in culture. **a**, The effect of murine LIF concentration on 8.5-d.p.c. PGC proliferation in culture. The number of PGCs present after 1 and 3 days of culture on confluent STO cell feeder layers. Bars represent the mean $\pm$ the standard deviation of five replicate cultures. Each experiment was done four times. **b**, The effect of bFGF concentration on 8.5-d.p.c. PGC proliferation in culture. The number of PGCs present after 1 and 3 days of culture on confluent STO cell feeder layers. Bars represent the mean $\pm$ the standard deviation of 5 replicate cultures. Each experiment was done four times.

METHODS. Cultures of 8.5-d.p.c. PGCs on confluent STO feeder cells were established from B6C3F1 animals as previously described^{4,7}. Recombinant murine LIF (ESGRO) and recombinant human bFGF were purchased from Gibco/BRL.

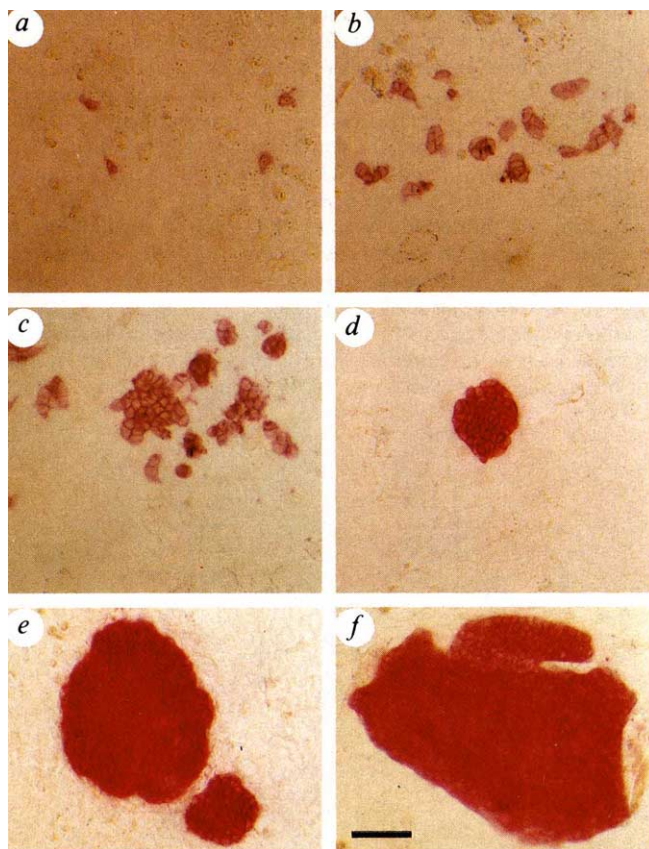


FIG. 2 Proliferation of 8.5-d.p.c. PGCs in bFGF and LIF and growth of EG cell colonies. *a*, PGCs, identified by alkaline phosphatase activity 1 day after isolation from 8.5-d.p.c. embryos and plating onto STO cell feeder layers in bFGF and LIF. PGCs exist as single cells. *b*, Three days after isolation PGCs are beginning to form small colonies, either by clonal division or aggregation. Some single PGCs are still identifiable. *c*, Five days after isolation PGCs are found in increasingly larger colonies. Many small colonies are found clustered near each other. *d*, Small colony of EG cells growing as a cellular monolayer on top of the STO feeder layer. *e*, Two colonies of EG cells. The smaller colony is growing as a monolayer, and the larger colony has become multilayered. *f*, Large, multilayered EG cell colony identified 9 days after plating PGCs onto confluent STO cell feeder layers. Scale bar, 100 μ m. METHODS. Cultures of PGCs were established from 8.5-d.p.c. embryos and PGCs identified by alkaline phosphatase histochemistry as previously described^{4,7}.

immortalization of PGCs, for three reasons. First, EG cells are not established in the absence of these factors (refs 4–7, and our unpublished data). Second, the rate of EG colony formation is significantly higher than the negligible rate of spontaneous PGC transformation seen in the B6C3F1 animals used in these studies (ref. 12, and H. Bedigian and J. Sundberg, personal communication). Third, the time course of EG derivation is very fast (9 days) compared to the time course of spontaneous immortalization of primary mouse embryonic fibroblasts in culture (3 months)¹⁵. Whether bFGF or LIF are expressed in the developing gonad and whether PGCs express an FGF receptor or an LIF receptor remain to be determined. Nevertheless, the ability

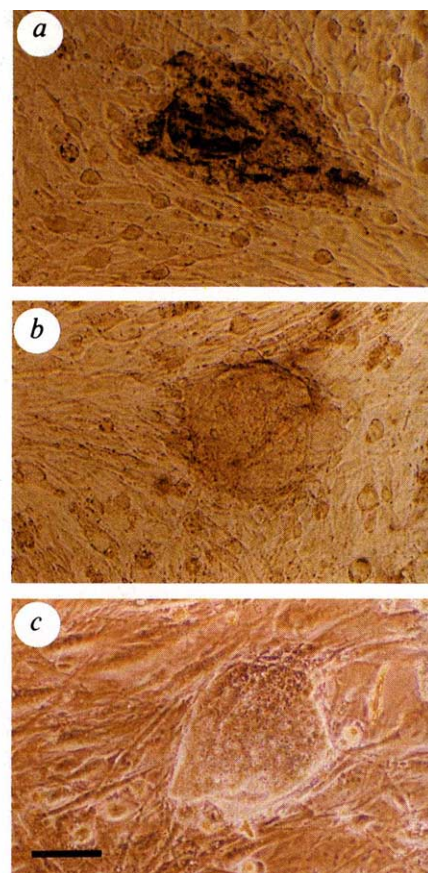


FIG. 3 Antigenic and morphologic characterization of PGC-derived EG cells. *a*, Surface staining of an EG cell colony with an anti-SSEA-1 antibody (IgM). Positive staining is detected as a peroxidase precipitate. STO cells and other embryonic somatic cells are negative⁷. *b*, Surface staining of EG cell colony with an isotype-matched (IgM) control antibody. No peroxidase staining is observed. *c*, Phase-contrast micrograph of an EG cell colony growing on top of the STO cell feeder layer. Scale bar, 100 μ m.

METHODS. Cultures of 8.5-d.p.c. PGCs were established as previously described^{4,7}. For immunocytochemistry, cultures were fixed with 4% paraformaldehyde in PBS, washed three times with PBS, and then incubated with primary (anti-SSEA-1) antibody (diluted in 10% heat-inactivated normal goat serum) for 1 h at room temperature. The cultures were then washed three times with PBS and then incubated with the peroxidase-conjugated secondary (goat anti-mouse polyvalent immunoglobulins) antibody for 30 min at room temperature. After antibody binding, cultures were washed with PBS and positive staining was identified by peroxidase histochemistry with DAB, hydrogen peroxide, cobalt and nickel chloride. Positive cells were stained dark brown.

of bFGF and LIF to stimulate long-term PGC proliferation provides a unique tool for the analysis of PGC development and studies of such key issues as recombination, pluripotency, sexual differentiation and imprinting.

Note added in proof: Since acceptance of this manuscript, Masui *et al.*¹⁶ have reported similar effects of bFGF and LIF on PGCs and have further shown that the derived cells form teratomas and contribute to chimaeras. □

Received 1 July; accepted 24 August 1992.

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ACKNOWLEDGEMENTS. We thank R. M. Frederickson, G. T. Smith and P. J. Donovan for their help, C. M. Hahn for typing the manuscript, and M. A. Bedell, C. I. Brannan and L. F. Lock for reading the manuscript and for their help and advice, and C. L. Stewart, N. G. Copeland and N. A. Jenkins for discussions and encouragement. This work was supported in part by the National Cancer Institute, DHHS.

Apoptotic cell death induced by *c-myc* is inhibited by *bcl-2*

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APOPTOSIS is a form of physiological cell death, characterized by chromatin condensation, cytoplasmic blebbing and DNA fragmentation¹, which often depends on RNA and protein synthesis by the dying cell²⁻⁴. The *c-myc* proto-oncogene, usually implicated in cell transformation, differentiation and cell-cycle progression⁵⁻⁹ also has a central role in some forms of apoptosis¹⁰⁻¹³. These opposing roles of *myc* in cell growth and death require that other gene products dictate the outcome of *c-Myc* expression on a cell. A candidate for such a modifying gene is *bcl-2*, whose product prolongs cell survival¹⁴⁻¹⁶ and blocks apoptosis in some systems¹⁷⁻²¹. Here we demonstrate that Bcl-2 prevents apoptotic death induced by *c-Myc*, provide a mechanism whereby cells can express *c-Myc* without undergoing apoptosis, and give a possible explanation for the ability of Bcl-2 to synergize with *c-Myc* in cell transformation²¹⁻²³.

To determine whether Bcl-2 prevents *c-Myc*-induced apoptosis we took advantage of a system using Chinese hamster ovary (CHO) cells to express an amplified *c-myc* construct under the control of a heat-shock promoter^{24,25}. Heat shock-inducible overexpression of *c-Myc* in these cells was demonstrated, rather than the decrease in *c-Myc* expression normally associated with heat shock²⁶. This elevated *c-Myc* expression resulted in cell death^{24,25}. Two sublines of these cells, designated 5A100 and 5A300, were transfected with human *bcl-2* on a constitutive promoter (pSFFV *bcl-2*nl; ref. 19). Incubation for 1 h at 43 °C

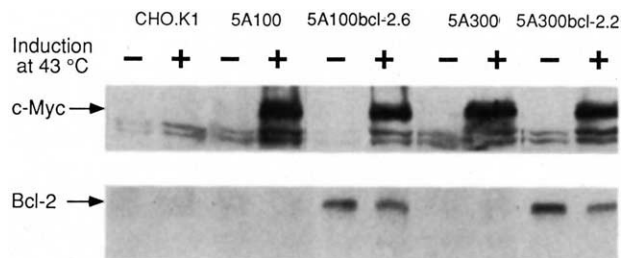


FIG. 1 Western blot analyses of *c-Myc* and Bcl-2 expression in parental and pSFFV *bcl-2*nl transfected 5AHSmyc cells before (–) and after (+) induction of *c-myc* expression by heat shock.

METHODS. 5AHSmyc cells, containing an amplified *c-myc* gene under control of a heat-shock promoter¹⁸, and the control line CHO.K1, were obtained from the American Type Culture Collection. Sublines 5A100 and 5A300 were derived from the 5AHSmyc line. The cells were transfected with the plasmid pSFFV *bcl-2*nl¹⁷, which contains a spleen focus-forming virus long terminal repeat driving the constitutive expression of a human *bcl-2* complementary DNA. Two *bcl-2*-expressing clones, 5A100 *bcl-2*.6 and 5A300 *bcl-2*.2 were used primarily. Cells expressing Bcl-2 were morphologically indistinguishable from the parental lines, and had similar doubling times (ranging from 20.2 to 24.4 h, with no significant differences seen). Induction of *c-myc* was by immersion in a 43 °C water bath (heat shock) for 90 min, after which the cells were incubated for further 6 h at 37 °C. The cells were recovered by trypsinization, and the protein prepared for analysis as described²⁹. Equivalent amounts of protein were solubilized in sample buffer with 2-mercaptoethanol and separated by SDS-PAGE. The gel was blotted and the relevant protein detected with either monoclonal anti-mouse *c-Myc* antibody (clone 152.6D11, NCI/BCB repository) or monoclonal anti-human Bcl-2 antibody (clone 6C8, provided by S. Korsmeyer), followed by biotinylated protein A and Extravidin-alkaline phosphatase (Sigma). Endogenous hamster *c-Myc* expression as not detected with this antibody.

induced equivalent levels of *c-Myc* (Fig. 1) in 5A100, 5A300, and in the *bcl-2*-transfected lines 5A100 *bcl-2*.6 and 5A300 *bcl-2*.2. The *bcl-2* transfectants produced Bcl-2 before and after heat-shock treatment. Levels of *c-Myc* correlated with heat-shock interval, and were detectable ~1 h after returning the cells to 37 °C, peaking at about 5–6 h after heat-shock treatment (data not shown) as described^{24,25}. The presence of Bcl-2 markedly influenced cell viability after *c-myc* induction (Fig. 2). Cell lines containing the inducible *c-myc* (5A100, 5A300) died, as determined in a short-term viability assay (Fig. 2a) or in a clonogenicity assay (Fig. 2b), whereas cells expressing Bcl-2 were relatively resistant to *c-myc*-induced cell death.

DNA fragmentation is a characteristic feature of apoptosis¹. Increased DNA fragmentation resulted from *c-myc* induction for increasing periods of time (30–120 min) in the lines without

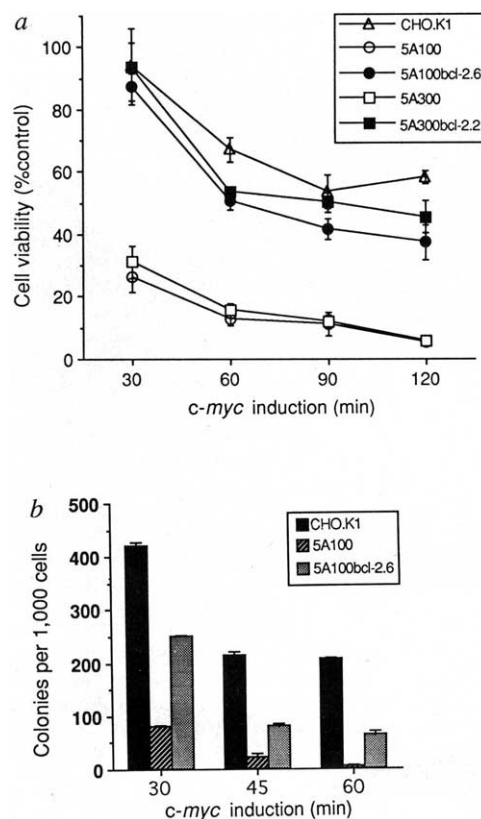


FIG. 2 The relative survival of parental 5AHSmyc (5A100, 5A300) and *bcl-2*-transfected 5AHSmyc cells (5A100bcl-2.6, 5A300bcl-2.2) after *c-myc* induction by heat shock for different periods of time. Cells were examined for: *a*, cell viability (number of viable cells expressed as a percentage of unheated control(s) and *b*, clonogenicity (number of colonies arising per 1,000 cells seeded). Results represent the mean of either quadruplicate (*a*) or triplicate (*b*) cultures with standard deviation (bars). Plating efficiency of the cell lines in the absence of heat shock was as follows: CHO.K1, 85 ± 8.2%; 5A100, 99.3 ± 6.6%; 5A100bcl-2.6, 89.2 ± 46%. Another transfected cell line, 5A100bcl-2.23, which failed to express detectable levels of Bcl-2 after selection in G418, lost clonogenic potential in a manner similar to 5A100 (data not shown).

METHODS. The effects of *c-myc* induction by heat shock were determined as follows. On the previous day, the cells were trypsinized, seeded into either 25-cm² flasks (1 × 10⁶) or 24-well plates (1 × 10⁵). The cells were heat shocked to induce *c-Myc* expression by immersion in a 43 °C water bath for the times indicated on the x-axis. After heat shock, the media was changed and the cells incubated at 37 °C. Cell viability was determined 24 h later by trypan-blue exclusion. For the clonogenicity assay, the cells were heat shocked for 30, 45 or 60 min, the cells dispersed by trypsinization and seeded onto 100-mm diameter dishes. Colony formation was assessed on day 10 by counting individual colonies after staining with 0.04% crystal violet in 20% ethanol.

Bcl-2 (Fig. 3a). This had little effect on one of the Bcl-2-expressing lines (5A100 bcl-2.6), whereas in the other (5A300 bcl-2.2), the protective effect of Bcl-2 diminished with increased time of *c-myc* induction. After heat induction for 60 min, cells expressing *c-Myc* (without Bcl-2) displayed increasing DNA fragmentation over the next 15 h, whereas the constitutive expression of Bcl-2 prevented DNA fragmentation at all times sampled (Fig. 3b). The fragmented DNA showed the distinctive pattern of oligonucleosomes found in apoptotic cells (Fig. 3c).

The effects of Bcl-2 were examined in several different clones (Table 1). Although all clones expressed comparable levels of *c-Myc* after heat shock, they differed in their susceptibility to *c-Myc*-induced DNA fragmentation. All clones expressing Bcl-2 were resistant to the *c-myc*-induced DNA fragmentation, whereas those lacking detectable Bcl-2 were susceptible.

An interleukin-3 (IL-3)-dependent cell line, transfected with a constitutively expressed *c-myc* gene, underwent apoptosis more rapidly on removal of IL-3 than the parental line¹¹. Similarly, *c-myc*-transfected Rat-1 cells died by apoptosis at low

TABLE 1 *c-Myc* and Bcl-2 expression compared with DNA fragmentation in *bcl-2*-transfected clones

Cell line	<i>c-Myc</i>	Bcl-2	Apoptosis/per cent fragmentation	
			Experiment 1	Experiment 2
CHO.K1	—	—	1.5	ND
5A100	+	—	49.4	35.4 ± 4.6
5A300	+	—	28.5	58.8 ± 6.1
<i>bcl-2</i> transfectants				
5A100bcl-2.6	+	+	7.8	3.6 ± 2.9
5A100bcl-2.9	+	+	4.8	5.3 ± 4.2
5A300bcl-2.1	+	+	0.0	6.4 ± 1.6
5A300bcl-2.2	+	+	7.1	0.0 ± 1.5
5A100bcl-2.3	+	—	58.4	ND
5A100bcl-2.11	+	—	51.3	ND
5A100bcl-2.23	+	—	44.4	46.7 ± 3.7
5A300bcl-2.7	+	—	18.3	32.6 ± 4.1
5A300bcl-2	+	—	ND	22.4 ± 10.0
5A300bcl-2.11	+	—	ND	34.3 ± 10.0

The induction of *c-myc*, and the analysis of *c-Myc* and Bcl-2 expression in individual clones by western immunoblot, as described in Fig. 1. DNA fragmentation after *c-myc* induction was determined using ³H-TdR labelled cells as described in the legend to Fig. 3. Each data point in experiment 1 represents a single measurement from three pooled cultures. Each data point in experiment 2 represents the mean of triplicate samples ± standard deviation. The difference in DNA fragmentation between the Bcl-2⁺ and the Bcl-2⁻ clones is significant (experiment 1, ANOVA, *P* = 0.005; experiment 2, ANOVA, *P* = 0.001). ND, not determined.

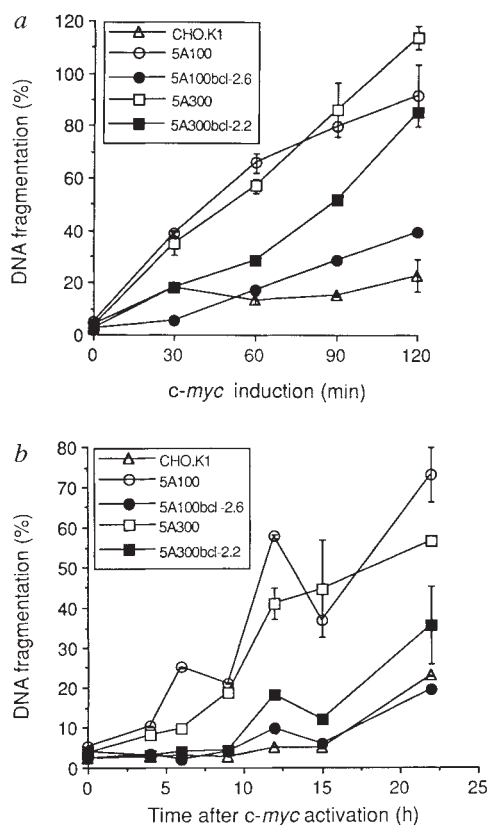
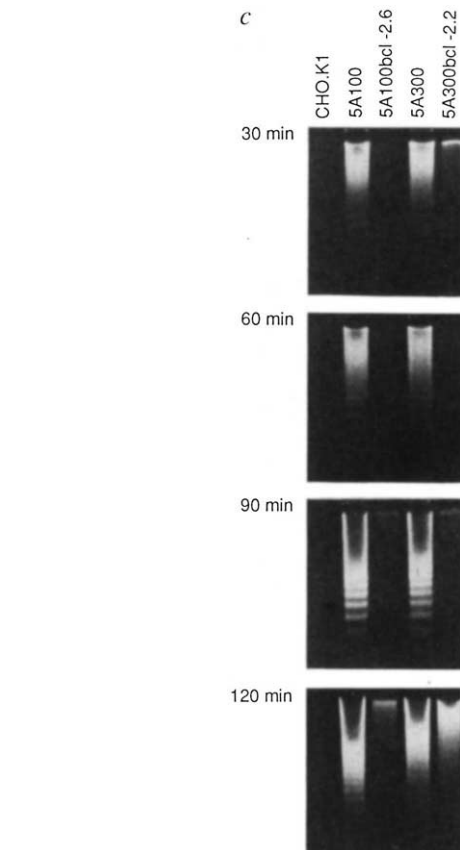


FIG. 3 The kinetics of *c-myc*-induced DNA fragmentation in parental and *bcl-2*-transfected 5AHSmyc cells. *a*, DNA fragmentation after *c-myc* induction for various periods of time as determined 20 h post induction. *b*, The kinetics of DNA fragmentation following *c-myc* induction for 60 min as determined at various times post-induction. *c*, Agarose gel analyses of DNA fragmentation following various induction times.

METHODS. The cells were prepared as described in Fig. 1, with the exception that ³H-TdR label was added for 8 h before the induction of *c-myc* by heat shock. The cells were heat shocked for the indicated interval (*a*, x-axis or *b*, 60 min) and then incubated at 37 °C for the indicated time (in *b*, x-axis or *a*, 20 h). The cells were recovered by trypsinization, and the cells lysed in 5 mM Tris-HCl pH 8, 10 mM EDTA, 0.5% Triton X-100. The lysate was centrifuged for 20 min at 13,000*g* to separate the fragmented DNA (soluble) from intact chromatin (pellet)¹⁴. DNA fragmentation is expressed as the amount of soluble DNA recovered as a percentage of total c.p.m. Results represent the mean of triplicate determinations with standard deviation (bars). Samples for analysing DNA fragmentation by agarose gel electrophoresis were obtained from cells that had been heat shocked for 30–120 min (as indicated) and then incubated at 37 °C for 16 h. The DNA samples were prepared as above, except that soluble DNA was treated with



RNAase A (100 µg ml⁻¹) for 1 h at 37 °C, followed by proteinase K (200 µg ml⁻¹) in 1% SDS for 2 h at 50 °C. The DNA was extracted sequentially with phenol, phenol/chloroform and precipitated in ethanol before electrophoresis on 1.2% agarose gels.

but not at high levels of serum. Thus, without a second survival signal, such as that provided by growth factor receptor interaction, c-Myc induces apoptosis. But if such a second signal is present, the cells are allowed to progress through the cell cycle. A 'two signal' model emerges, in which c-Myc can provide the first signal, leading either to apoptosis or to progression, and certain growth factors may provide a second signal, to inhibit apoptosis and allow c-Myc to drive cells into the cell cycle. Bcl-2 may substitute for the putative survival signal, as suggested by the abilities of Bcl-2 to delay apoptosis after growth-factor withdrawal^{18,21} and to cooperate with c-Myc in transforming cells²¹⁻²³. In primary cells transfected with c-myc, there is often a period of extensive cell death before the appearance of transformed cells²⁷, which may represent apoptosis and subsequent selection for the endogenous activation of a second signal²⁸. □

Received 18 May; accepted 18 August 1992.

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ACKNOWLEDGEMENTS. We thank D. Hockenberry and S. Korsmeyer for the pSFFV bcl-2nl construct and the monoclonal anti-Bcl-2 antibody, and W. Nishioka and A. Fotedar for discussion. This research was supported by the National Institutes of Allergy and Infectious Disease, and by Gemini Science.

Cooperative interaction between c-myc and bcl-2 proto-oncogenes

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THE *bcl-2* proto-oncogene is activated by translocation in a variety of B-lymphoid tumours and synergizes with the *c-myc* oncogene in tumour progression¹. The mechanism of synergy is unclear but *bcl-2* expression inhibits apoptosis²⁻⁶, a property presumably pertinent to its proto-oncogenic mode of action⁴. We have shown that the *c-myc* gene is a potent inducer of apoptosis, in addition to its established role in mitogenesis⁷. Here we show that expression of the *bcl-2* protein, Bcl-2, specifically abrogates c-Myc-induced apoptosis without affecting the c-Myc mitogenic function. This

provides a novel mechanism for oncogene cooperation, of potential importance both in carcinogenesis and in the evolution of drug resistance in tumours.

Rat-1 fibroblasts that constitutively express a chimaeric protein, comprising a full-length c-Myc polypeptide fused to part of the human oestrogen receptor (Rat-1/c-Myc-ER cells), show demonstrable c-Myc activity only in the presence of β -oestradiol⁷⁻⁹. Thus, in the absence of β -oestradiol, serum-deprived Rat-1/c-Myc-ER cells arrest in a stable G0-like state, whereas addition of β -oestradiol prevents arrest and induces rapid apoptotic death⁷. This apoptosis is dependent on c-Myc expression; its extent is proportional to the level of intracellular c-Myc protein, and it requires the same regions of the c-Myc

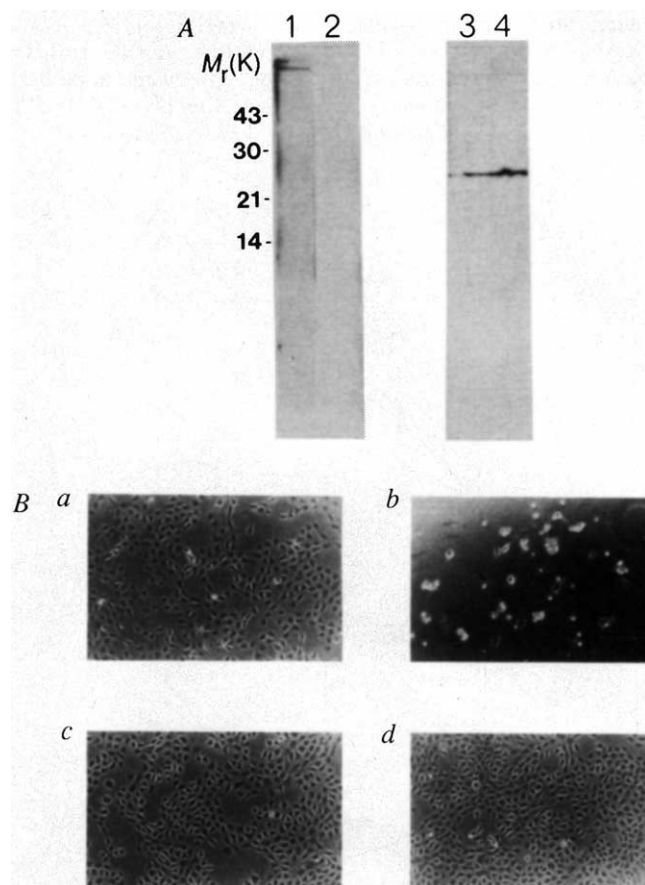


FIG. 1. A, Determination of expression of Bcl-2 α protein by immunoblotting. Lysates from control Rat-1/c-Myc-ER cells (lanes 1 and 2) and a representative Rat-1/c-Myc-ER/Bcl-2 clone (clone 5) (lanes 3 and 4) were fractionated on a 15% SDS-polyacrylamide gel, blotted onto nitrocellulose paper and probed with either mouse (Bcl-2/100; lanes 1 and 3) or a hamster (6C8; lanes 2 and 4) anti-Bcl-2 monoclonal antibodies. B, Constitutive Bcl-2 expression prevents c-Myc-induced apoptosis in serum-deprived Rat-1 fibroblasts. Various Rat-1-derived cell lines were transferred into medium containing 0.05% fetal calf serum either with or without 2 μ M β -oestradiol and observed after 48 h. a, Rat-1/c-Myc-ER, no β -oestradiol (c-Myc inactive), b, Rat-1/c-Myc-ER plus β -oestradiol (c-Myc active), c, Rat-1/c-Myc-ER/Bcl-2, no β -oestradiol, d, Rat-1/c-Myc-ER/Bcl-2 plus β -oestradiol.

METHODS. Production and maintenance of Rat-1 fibroblasts stably expressing the c-Myc-ER fusion protein and the defective c-Myc mutant protein $\Delta 106-143$ c-Myc-ER have been described⁷. A retrovirus vector directing constitutive expression of Bcl-2 α was made by expressing a full-length *bcl-2* α complementary DNA in pBabe PURO²⁰. Infectious ecotropic Bcl-2/PURO virus was prepared from culture supernatants of Ω E cells^{20,21}, transfected with the Bcl-2/PURO vector and this was used to infect Rat-1/c-Myc-ER cells²¹. Infected clones were selected in 5 μ g ml⁻¹ puromycin. Immunoblotting of bulk cultures of puromycin-resistant cells was as described²², using either the Bcl-2/100 mouse monoclonal antibody¹¹ (gift from D. Y. Mason) or the 6C8 hamster monoclonal antibody³ (gift from S. J. Korsmeyer).

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protein as are required for co-transformation and autosuppression⁷. The role of c-Myc in apoptosis is not confined to fibroblasts, having also been described in haematopoietic cells¹⁰.

To investigate the effect of *bcl-2* expression on c-Myc-induced apoptosis, we infected Rat-1/c-Myc-ER cells with a retrovirus directing constitutive expression of human *bcl-2* α together with a puromycin-resistant marker. Puromycin-resistant cells that expressed Bcl-2 protein (Rat-1/c-Myc-ER/Bcl-2 cells) were identified by immunoblotting (Fig. 1a) and immunocytochemistry (data not shown) using anti-Bcl-2 monoclonal antibodies^{3,11}. Proliferating Rat-1/c-Myc-ER/Bcl-2 fibroblasts and control cells were then assayed for c-Myc-induced apoptosis by microscopic inspection 48 h after serum depletion. Constitutive Bcl-2 expression effectively abolished c-Myc-induced apoptosis under these conditions (Fig. 1b). Essentially identical results were observed with 10 individual clones and one, clone 5, was the example used here. To quantify more accurately the inhibition of c-Myc-induced apoptosis by Bcl-2, we caused Rat-1/c-Myc-ER and Rat-1/c-Myc-ER/Bcl-2 cells to arrest in phase G0 by serum depletion and then activated c-Myc by addition of β -oestradiol. This induced the rapid onset of apoptosis which can be quantitated by time-lapse cinemicroscopy⁷. Time-lapse cinemicroscopy also enables each death to be confirmed as apoptotic: apoptosis is characterized by its extreme rapidity, taking about 30–60 min to proceed through a diagnostic sequence of cytoplasmic blebbing, vesicularization and nuclear condensation^{12,13}. Expression of Bcl-2 completely inhibits the onset of apoptosis in serum-deprived Rat-1 cells after c-Myc activation, as shown in Fig. 2.

It is important to determine whether Bcl-2 blocks c-Myc-induced proliferation as well as apoptosis: the synergy between c-Myc and Bcl-2 can easily be rationalized if Bcl-2 blocks only the apoptotic function of c-Myc and leaves its mitogenic activity unaffected. We examined the mitotic rate of Rat-1/c-Myc-ER cells that were proliferating in low serum under the influence of β -oestradiol-activated c-Myc, with and without Bcl-2 expression. A proportion of the cells without Bcl-2 died before

undergoing division, as previously described⁷, and thus could not be scored. We excluded such cells from our analysis and studied 100 of the remaining cells, chosen at random, through their first divisions. The results (Fig. 3) show that the mitotic rates of cells are similar, irrespective of Bcl-2 expression, demonstrating that Bcl-2 expression does not prevent cell proliferation. Bcl-2 does not, therefore, inhibit all c-Myc functions but specifically blocks c-Myc-induced apoptosis.

Deregulated c-Myc expression induces the rapid onset of apoptosis in cells arrested by a variety of cytostatic and cytotoxic drugs⁷. Many primary tumours also respond to such drugs by initiating apoptosis¹⁴ and, because *myc* gene activation is so widespread in human tumours¹⁵, we have suggested that this is also c-Myc-induced⁷. If we are correct, this implies that lesions in the apoptotic pathway, such as Bcl-2 activation, might contribute towards drug resistance in tumours. We therefore investigated the effect of Bcl-2 expression on c-Myc-induced apoptosis in Rat-1/c-Myc-ER cells arrested either with thymidine, which causes a block in the S phase, or with the epipodophyllatoxin etoposide (VP16), a topoisomerase II inhibitor that arrests cells in S/G2 and is frequently used in cancer chemotherapy¹⁶. In both cases, Bcl-2 expression substantially delayed and reduced c-Myc-induced apoptosis (Fig. 4), effectively increasing the resistance of cells to each drug. In neither case did Bcl-2 expression overcome the cytostatic effect of the drugs (data not shown).

Deregulated expression of c-Myc in tumour cells is extremely common, indicating that its acquisition may be essential during carcinogenesis. But the potency with which c-Myc induces apoptosis suggests that deregulated c-Myc expression by itself is likely to be lethal because it kills any cell that encounters growth-limiting conditions, an almost invariable outcome *in vivo*. In such a situation, any additional mutation that blocks apoptosis will strongly promote survival of the affected cell and so allow its continued proliferation, mutation and carcinogenic evolution. Our results demonstrate that acquisition of constitutive Bcl-2 expression is an example of such a mutation. Bcl-2

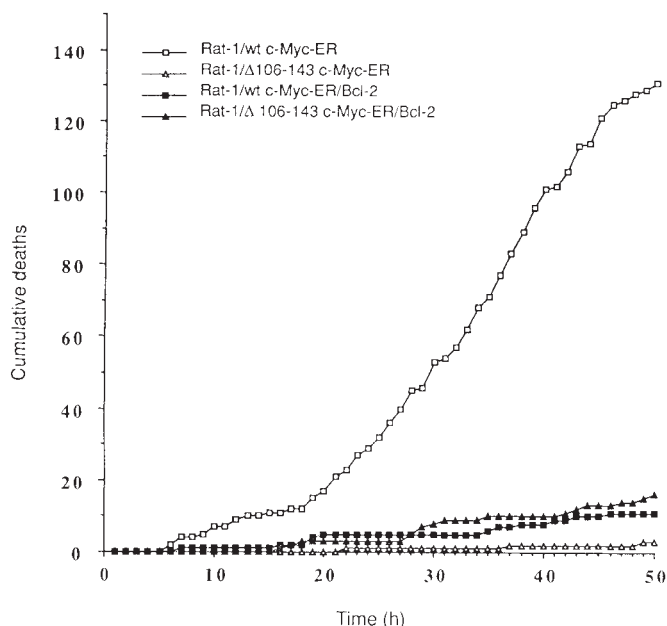


FIG. 2 Time-lapse cinemicroscopic quantitation of apoptosis in Rat-1 cells. Rat-1 cells expressing either wild-type c-Myc-ER or the defective c-Myc mutant $\Delta 106-143$ fused to the human oestrogen receptor, and either with or without Bcl-2, were growth-arrested by culture in 0.05% fetal calf serum (FCS) for 48 h. Activation of c-Myc was by addition of β -oestradiol (2 μ M) to the culture medium, and cells were observed by time-lapse cinemicroscopy. The cells did not reach confluence. Apoptotic cell deaths were scored as described⁷ and cumulative deaths plotted against time. Each field started with 95 cells and images were taken at a rate of 12 frames per hour.

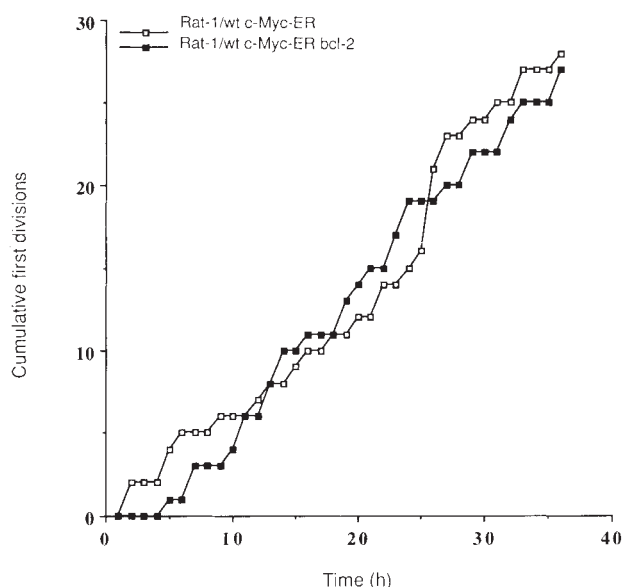
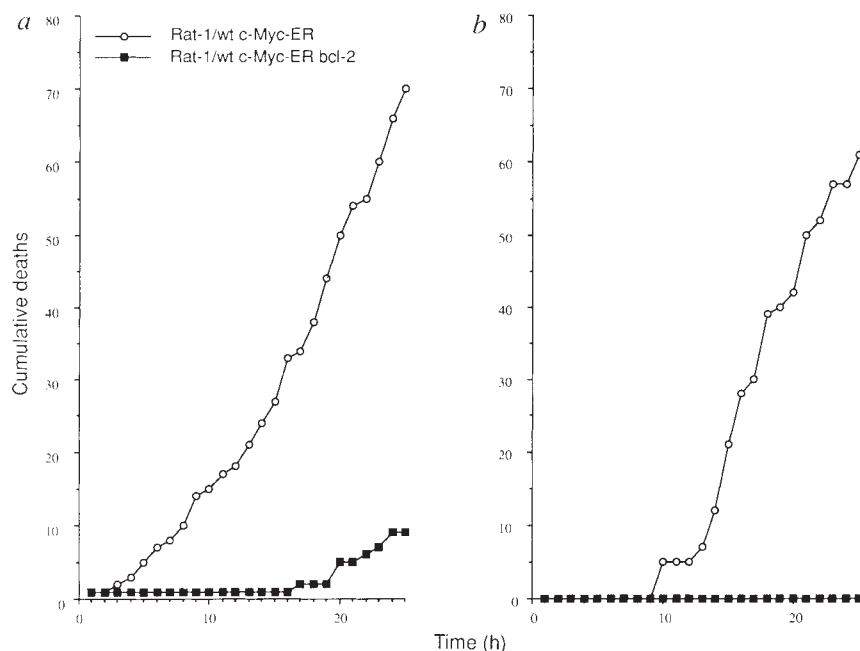


FIG. 3 Time-lapse cinemicroscopic analysis of the effect of Bcl-2 expression on c-Myc-induced proliferation of Rat-1 cells. Rat-1/c-Myc-ER and Rat-1/c-Myc-ER/Bcl-2 cells were cultured in 0.05% FCS containing 2 μ M β -oestradiol, and mitotic events observed by time-lapse cinemicroscopy at 12 frames per hour. Cells that underwent apoptosis before division were excluded from the analysis. Of the remaining cells, 95 were randomly picked and their fates followed. Only first divisions in each lineage were counted to allow comparison between experiments. Cumulative divisions are plotted against time.

FIG. 4 Constitutive Bcl-2 expression protects Rat-1/c-Myc cells from thymidine and VP16/etoposide-induced apoptotic cell death. **a**, Thymidine block. Exponentially growing Rat-1/c-Myc-ER and Rat-1/c-Myc-ER/Bcl-2 cells were arrested in S phase by addition of 2 mM thymidine to the growth medium for a period of 24 h as described⁷. c-Myc was then activated by addition of β -oestradiol to a final concentration of 2 μ M and the cells were monitored by time-lapse cinematography at a rate of 12 frames per hour. Cumulative apoptotic deaths, of 100 cells of each type, are shown plotted against time. **b**, Etoposide/VP16 block. Exponentially growing Rat-1/c-Myc-ER and Rat-1/c-Myc-ER/Bcl-2 cells in 10% FCS were incubated for 24 h with 0.1 μ M etoposide/VP16 (Sigma). Activation of c-Myc and cinematography are described in **a**.



mitigates the apoptotic effects of deregulated c-Myc expression without affecting its ability to promote continuous cell growth, so providing a mechanistic basis for the oncogenic synergy between these two proto-oncogenes. The interaction between c-Myc and Bcl-2 differs from the usual form of oncoprotein cooperation as observed between c-Myc and activated Ras¹⁷ in that, although c-Myc/Bcl-2 fibroblasts proliferate continuously in the absence of mitogens, they neither appear to be morphologically transformed nor to form foci in monolayer culture¹⁸ (Fig. 1). The interaction between c-Myc and Bcl-2 thus represents a novel type of oncogene cooperation, undetectable by classical transformation-focus assays. Further characterization of the growth and death of cells containing various combinations of activated *c-myc*, *ras* and *bcl-2* oncogenes should provide insights as to how the various attributes of these three classes of oncogene interact.

The emergence of drug-resistant mutations in tumours reflects the fact that carcinogenesis is a continuous evolutionary process, often exacerbated by the increased genetic instability of cancer cells. Our results indicate that, just as activated c-Myc expression may be important in determining the initial sensitivity of tumours to drugs, so Bcl-2 deregulation, by blocking programmed cell death, may lead to drug resistance. This is a mechanism of drug resistance distinct from amplification and multidrug-resistance described previously¹⁹. The interaction between c-Myc and Bcl-2 thus represents a novel model for oncogene cooperation which has implications for the genesis and the progression of neoplastic disease. □

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ACKNOWLEDGEMENTS. We thank C. Gilbert and D. Davies for help with time-lapse cinematography and flow cytometry, respectively, and H. Land, M. Jacobson, M. Collins, P. Smith, T. Littlewood, D. Hancock and R. Treisman for advice and comment.

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All manuscripts should be typed, double-spaced, on one side of the paper only. An original and three copies are required, each accompanied by artwork. If photographs are included, four sets of originals are required: for line drawings, one set of originals and three good-quality photocopies are acceptable. Reference lists, figure legends and tables should all be on separate sheets, all of which should be double-spaced and numbered. Relevant manuscripts in press or submitted for publication elsewhere should be included with each copy of a submitted manuscript and clearly marked as such. Revised and resubmitted manuscripts should also be clearly marked as such and labelled with their manuscript numbers.

Titles say what the paper is about with the minimum of technical terminology and fewer than 80 characters in the case of Articles and Letters. Active verbs, numerical values, abbreviations and punctuation are to be avoided. Titles should contain one or two key words for indexing purposes.

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Protein/nucleotide sequences should ideally be in the three-letter and not the single-letter code for amino acids. One column width of *Nature* can accommodate 20 amino acids or 60 base pairs. Numbering of sequences should be in the left-hand margin only, with a single space between rows.

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References are numbered sequentially as they appear in the text, followed by those in tables and finally by those in figure legends. Only papers published or in the press are numbered and included in the reference list. All other forms of reference, including unlettered abstracts, should be cited in the text as a personal communication, manuscript submitted or in preparation. Text is not included in reference lists. References are abbreviated according to the *World List of Scientific Periodicals* (Butterworths, London, 1963–65). The first and last page numbers are included; reference to books should include publisher, place and date.

Abbreviations. Symbols, units and Greek letters should be identified the first time they are used. Acronyms should be avoided whenever possible and, if used, defined. Footnotes are not used except for changed addresses.

Acknowledgements are brief; grant and contribution numbers are not allowed.

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Received 3 June; accepted 19 August 1992.

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Competitive PCR

Paul D. Siebert and James W. Larrick

Competitive reverse transcription-polymerase chain reaction (RT-PCR) can be used to obtain quantitative information of mRNA levels comparable to traditional RNA blot techniques, with the added advantages of PCR.

THE combined use of reverse transcription followed by the polymerase chain reaction (RT-PCR) enables the amplification of individual RNA molecules. This method (recently reviewed in ref. 1) is variously termed RT-PCR², RNA-PCR³, RNA phenotyping⁴, and message amplification phenotyping⁵. RT-PCR has been shown to be 1,000–10,000 fold more sensitive than the traditional RNA blot techniques^{6–8}. The exquisite sensitivity of RT-PCR makes it possible to detect mRNAs of extremely rare abundance, mRNAs in small numbers of cells or in small amounts of tissue, and mRNAs expressed in mixed-cell populations. The expression of multiple mRNAs can be determined simultaneously, a difficult task by traditional methods. Additionally, the RT-PCR procedure requires only a few hours to perform.

Although RT-PCR has many advantages over RNA blot methods, it can be difficult to obtain quantitative information. This is due to the exponential nature of the PCR amplification, where small variations in amplification efficiency result in dramatic changes in product yields. In addition, the amount of product generated plateaus during later stages of the reaction due to consumption of necessary components and generation of inhibitors. These characteristics of PCR can obscure differences in the initial amounts of target sequences during the course of amplification. However, these problems can be overcome by the use of internal controls in the PCR.

One form of internal control utilizes a second set of primers in the same PCR tube designed to amplify a ubiquitously and constitutively expressed mRNA.

Housekeeping genes such as β_2 microglobulin, actins, and the enzymes dihydrofolate reductase and glyceraldehyde phosphate dehydrogenase have been used for this purpose. Unfortunately, mRNA levels of these genes do not always remain constant^{9–11}.

Although co-amplification of a control gene can correct for variation in tube-to-tube amplification efficiency these data must be obtained during the exponential phase of the reaction. Frequently, the reaction has already started to plateau when PCR products are first visualized on a gel stained with ethidium bromide¹². In this case, it is necessary to monitor PCR products by a more sensitive method using radioactively-labelled nucleotides or primers or by Southern blotting followed by probe hybridization. Obtaining data during the exponential phase of the reaction can be particularly

difficult to achieve if the control gene is expressed at a different level than the target gene. In addition, multiple sets of primers in the same tube often interfere with amplification of either the target gene, control genes, or both (P. D. Siebert, unpublished data). Competitive RT-PCR overcomes many of these difficulties.

Competitive RT-PCR

In competitive PCR, a DNA fragment containing the same primer template sequences as the target competes for primer binding and amplification. The PCR products are distinguished by size, hybridization, or change in a restriction site.

Becker-André and Hahlbrock¹³ and Gilliland *et al.*¹⁴, first described competitive PCR. Gilliland *et al.* utilized either a genomic fragment of the target gene containing a small intron (thus yielding a PCR product slightly larger than the target mRNA) or an engineered target cDNA in which a unique restriction site was added. In the latter case, PCR products were digested with a restriction enzyme before electrophoresis. Becker-André and Hahlbrock utilized a synthetic mRNA engineered to contain a unique restriction site.

The use of internal controls that contain the same primer template sequences as the target makes it possible to determine the absolute amount of target cDNA by allowing known amounts of competitor DNAs to compete with the target for primer binding during the amplification. In the usual case, known molar quantities of the competitor DNA are spiked into a series of PCR reaction tubes containing equal amounts of the target cDNA. Following PCR, the amount of products generated by the control and target are compared. The amount of competitor DNA yielding equal molar amounts of products gives the initial amount of target gene.

Other types of competitive DNA fragments can be utilized that have the same primer template sequence but a completely different intervening sequence. Uberla¹⁵ prepared competitive fragments by amplifying genomic DNA fragments from another species with low annealing stringency. Siebert and Larrick¹⁶ ligated the primer template sequences to a restricted DNA fragment in the form of an adaptor. More simply, the competitor DNA can be obtained by amplification of a foreign DNA fragment using two composite primers. Each composite primer has the target gene primer sequence attached to a short stretch of nucleotides,

which hybridize to opposite strands of the DNA fragment. The primer sequences are then incorporated into the PCR products during amplification (P. Kitts, unpublished data).

All competitive PCR methods require that the target gene and competitive fragment amplify with equal efficiencies. The use of a genomic DNA fragment with a small intron or a competitor fragment containing a single added restriction site is expected to amplify with equal efficiency. This may not be the case for heterologous competitor fragments. However, it has been our experience, and that of others⁸, that amplification efficiency is primarily determined by the primer sequences unless there are significant differences in denaturation or polymerase extension characteristics due to high G/C content or secondary structure.

Experimental studies

In the two experiments shown, competitive RT-PCR was used to study the induction of nitric oxide (NO) synthase mRNA in mouse macrophage cells by lipopolysaccharide (LPS). We prepared a primer pair and a

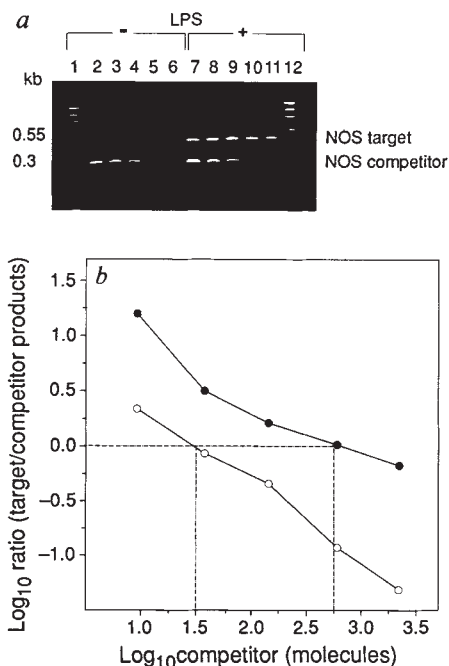


FIG. 1 Induction of nitric oxide (NO) synthase mRNA studied by competitive reverse transcription-polymerase chain reaction (RT-PCR). Four-fold serial dilutions of the competitor were co-amplified with constant amounts of cDNA. a, Agarose gel stained with ethidium bromide and b, logarithmic plot of these data.

heterologous competitor DNA fragment for NO synthase yielding PCR products of 550 and 300 base pairs, respectively. Figure 1 shows a competitor titration experiment with cDNA prepared from macrophage cells with and without LPS treatment for 6 hours. Equal portions of cDNA were amplified in the presence of 4-fold serial dilutions of the competitor. The products were then resolved on an agarose gel stained with ethidium bromide (a). The relative induction of NO synthase mRNA was determined by calculating how much of the competitor was required to achieve equal molar amounts of products. To do so, these data were quantitated by computer imaging of the Polaroid film (Ultraviolet Products, Inc., San Gabriel, California) and plotted (b). The amount of NO synthase cDNA was then calculated by extrapolating from the intersection of the curves, where the amounts of target and competitor are equal ($\log_{10} = 0$) to the x-axis. The values obtained, after correcting for the difference in size of the competitor and target, were 60 and 1,030 cDNA molecules, giving a change of 18 fold. It has recently been demonstrated by northern analysis¹⁷⁻¹⁹ that NO synthase mRNA is induced by at least 10 fold following treatment of macrophage cells with LPS for 6 hours in agreement with these results.

Figure 2 shows the time course for the induction of NO synthase mRNA studied by competitive RT-PCR. In this experiment, it would have been labour-intensive to perform a competitor titration for each time point, so instead equal amounts of competitor were included in each PCR reaction. In this way, it was still possible to correct for amplification efficiency in each tube and calculate relative fold induction. A maximum induction of about 26 fold was observed at 4 hours following LPS treatment.

Discussion

As the initial ratio of target-to-competitor cDNA remains constant throughout the amplification, it is not necessary to ob-

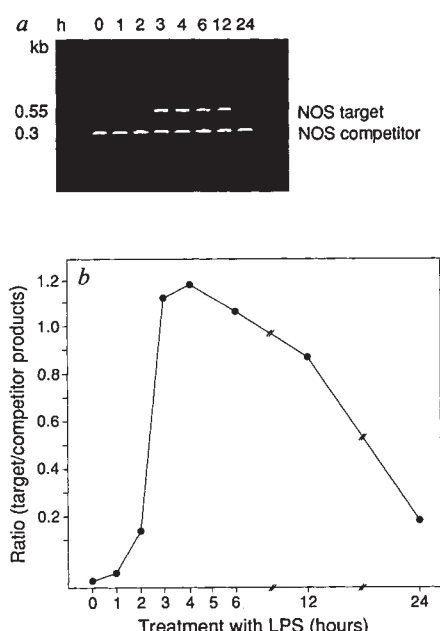


FIG. 2 Time course for induction of nitric oxide (NO) synthase mRNA studied by competitive reverse transcription-polymerase chain reaction (RT-PCR). A constant amount of competitor was co-amplified with the cDNA. a, Agarose gel stained with ethidium bromide and b, plot of the ratio of NO synthase to competitor PCR products.

tain data during the exponential phase of the reaction^{13,14,20}. This is perhaps the greatest advantage of using competitive PCR.

One potential disadvantage of the use of competitor DNA fragments in RT-PCR experiments is that there is no control for the efficiency of the RT-step. This has been addressed by Becker-André and Hahlbrock¹³ and Wang *et al.*⁸, who utilized competitor RNA fragments.

Using competitive RT-PCR, it is possible to obtain quantitative information of mRNAs comparable to RNA blot analysis. The ability to perform quantitative analysis of mRNAs by competitive

RT-PCR will undoubtedly expand the use and applications of PCR. □

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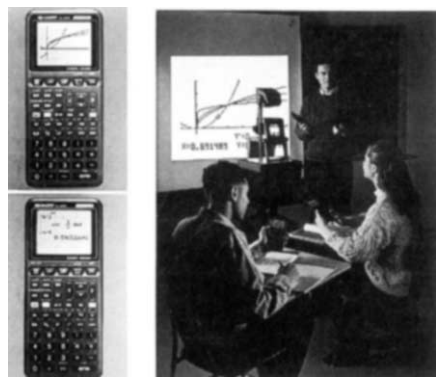
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Scientific gadgetry

Scientific graphing calculators that make light work of problem solving, a pulse controller to provide enhanced resolution of DNA samples and an imaging system for low-cost photography are featured this week.

SHARP Electronics has introduced a new line of **graphing calculators** that should be useful in applications ranging from algebra to calculus, statistics to the sciences (*Reader Service No. 101*). Two models are available: the EL-9200 and EL-9300. Both calculators feature high-contrast Super Twist LCD screens and user-friendly functions. An equation editor function enables equations to be entered, edited and viewed just as they appear in textbooks, rather than as a single line. The menu system provides users with fast access to a variety of mathematics functions and other operations. To simplify operation, four direct-access function keys with icons for quick reference allow functions to be changed instantly with one key stroke, the company says. For graphing applications, up to four equations can be graphed and analysed at once in rectangular, polar and parametric coordinates. To simplify statistics, applications are presented



Problem solving at the touch of a button.

in a 'card' format, with each card representing one data set or observation. Up to seven types of statistical graphs can be used to view statistical data. A programming language similar to BASIC stores up to 99 programs. The \$119.99 (US) EL-9200 has an 8 KB RAM memory; the \$149.99 (US) EL-9300 model has a 32 KB RAM memory. In addition, the EL-9300 has an equation solver, a backup battery and a communications port for communicating to an overhead projector (\$399.99 (US) model EL-92T), another calculator or a printer.

The new compact Model 910 **protein sequencer** from Knauer for the sequencing of peptides and proteins features a loop reactor that is specially designed for precise reagent delivery (*Reader Service No. 102*). Because of the small valves and integrated electronics, the Model 910 requires a minimum of bench space (width 87 cm). The microbore HPLC is an integral part of the

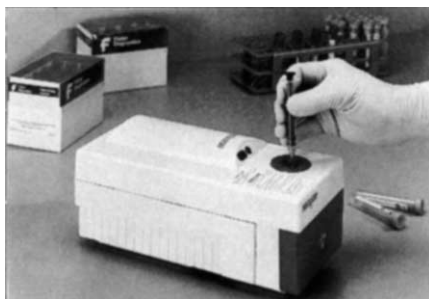


Knauer's Model 910 protein sequencer.

Model 910 and the detection of PTH derivatives lies in the femtomole range, according to Knauer. A full range of sequencing methods is offered, including sequence analysis of blotted, covalently attached and directly applied peptides and proteins at the low-picomole level. All sequencer and HPLC parameters are controlled and set via the PC running Microsoft Windows. The system includes a built-in CD-ROM with protein database and can be integrated into a network.

■ Safety measures

To help **prevent accidental needle-stick injuries** in pathogen-sensitive medical research, Fisher Scientific has introduced the Needlyzer — a benchtop device designed to convert a stainless steel needle into granules in seconds (*Reader Service No. 103*). A transformer produces a low voltage, high current across two tungsten electrodes to short out and destroy the needle. Although incineration takes place at 1,500 °C, the outer casing of the Needlyzer remains cool to the touch, Fisher Scientific says. Residue



Needlyzer needle incinerator.

is captured in the disinfectant-lined disposable cartridge, which is designed to hold 2,500–3,500 destroyed needles. Contents of the cartridge can be viewed through the transparent lid. The remaining syringe is discarded as hazardous waste. A safety interlock switch prevents operation unless the cartridge is fully inserted.

Northumbria Biologicals has introduced an automatic system for **sterilizing bacteriological inoculating loops**, which is designed to relieve staff of the tedious task of manual sterilization and to save energy by preventing unnecessary heating of ambient air (*Reader Service No. 104*). An optical sensor detects and sterilizes used loops and their securing nuts, which can often harbour bacteria. In this way, loops are sterilized effectively and without error, the company says.

■ DNA separations

Hoefer Scientific's SwitchBack **pulse controller** is programmable, portable and user friendly and is designed to enhance the resolution of DNA samples in the 2-kilobase (kb) to 2-megabase (Mb) range (*Reader Service No. 105*). Designed to fit into any conventional DNA electrophoresis system, the SwitchBack device allows

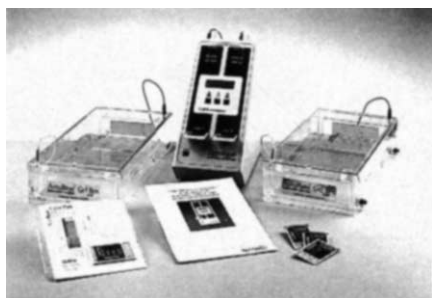


SwitchBack — Hoefer's resolution enhancer.

concatenation of individual programmes so that large and small DNA samples can be separated in one gel. Hoefer says that users need to enter only initial and end pulse duration and total run time, leaving the SwitchBack system to calculate all the necessary parameters. Users can store up to 60 protocols. In addition, Hoefer provides suggested protocols to cover the whole DNA size range (2 kb–2 Mb). The SwitchBack system should be of interest to researchers performing low-range genomic analysis (less than 1 Mb), non-forensic restriction fragment length polymorphism studies, cosmid mapping and YAC cloning.

AutoBase from Q-Life Systems, which is being distributed by TechGen, stands apart from conventional **pulsed-field electrophoresis systems** in that it utilizes a dual-channel power supply unit that is driven and controlled by pre-optimized software captured on ROM cards (*Reader Service No. 106*). TechGen says that the performance of the system is further enhanced by

using zero-integrated field electrophoresis techniques. Each ROM card contains all the necessary programmed information for DNA separations, allowing the user to optimize individual applications such as band analysis, purification and Southern blotting. The cards make up the GenePack library, which cover all of the main separation requirements from 8-kilobase pairs to 6-megabase pairs. TechGen will also supply customized protocols on ROM cards. The user decides on the DNA sizes of interest and the resolution required, chooses the appropriate GenePack ROM card and then lets the instrument do the rest — a method that is designed to remove the guesswork and eliminate time-consuming method



AutoBase pulsed-field electrophoresis system.

development and verification. The microprocessor-controlled power supply is designed to ensure reproducible power output from the dual channels. These allow either gel tray to be run independently, as duplicates or one at a time. TechGen says that this alone offers the potential to maximize throughput due to the extensive times required for such pulsed-field applications.

The IS-1000 digital imaging system from Innotech Scientific provides an instant photography, digital imaging and gel



All in one digital imaging system.

documentation system all in one complete system (*Reader Service No. 107*). The system, which, the company says, features the latest CCD video camera technology and powerful operating software, provides users with a low-cost means of producing photographic prints (prints cost 7–10 cents per print). It also offers image enhancement, image processing and image archiving functions. The operating software utilizes a mouse and onscreen controls for easy access to functions such as automatic and manual image enhancement for hard-to-see bands, annotation to label and mark various areas of the image, noise reduction filters, exposure, zoom and panning functions, positive or negative images and false colour for better viewing. Images are saved as TIFF files. An optical drive for mass storage is optional. In addition, quantitation and analysis software is available. Images can also be imported into word processing and desktop publishing software packages.

■ Under the microscope

The new EM 912 Omega **energy filter transmission electron microscope (TEM)** from Carl Zeiss features an integrated imaging spectrometer of the Omega-type which operates at accelerating voltages of up to 120 kV (*Reader Service No. 108*). Based on the technology used in the earlier EM 910, the EM 912 Omega offers dedicated multilayer management of all microscope functions and components and features a newly developed electron-optical column that provides considerable imaging capabilities. The imaging Omega spectrometer, which comprises four magnetic prisms and a hexapole corrector located in the plane of symmetry, is an integral part of the microscope's optical system. The entrance and exit planes are designed to be conjugate to the respective imaging and diffraction planes and remain fixed for all magnifications and diffraction lengths. This, Carl Zeiss says, eliminates the need for the user to align the system. In addition, the company says that inelastically scattered electrons, which markedly decrease the scattering and phase contrast of the final image in a conventional TEM, are eliminated by zero energy-loss filtering in the EM 912 Omega. The Omega filter remains effective throughout the entire magnification range. The EM 912 Omega, therefore, allows energy-filtered images or diffraction patterns of large or small specimen areas to be recorded without the need for time-consuming scanning. In the electron-spectroscopic imaging mode, the relationships between the ultrastructure of the specimen and the element distribution in the specimen become directly visible online by element contrast imaging.

Nikon's new **microphotometry system** supersedes the company's P1 model and should be of interest to researchers in the life and physical sciences (*Reader Service*

No. 109). The P101 photometer controller builds on the strength of the P1 in routine analysis, while the more sophisticated P102 has additional features, including measurement of alternating current (a.c.) and chopper shutter control, which make it suitable for accurate quantification. A range of accessories allows users to configure the equipment to suit most applications in quantitative microscope photometry. Whereas fluorescence photometry is likely to be the



Nikon's photometry system.

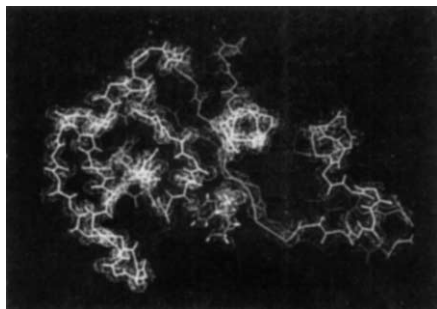
primary area of application, particularly in the field of immunology, Nikon believes that the instrument will also prove to be useful in absorption photometry (as used in many life science disciplines) and in reflectance photometry (as used in mineralogy, geology, semiconductor research, petrology, the physical sciences and forensic science). New features of this upgraded system include a variety of finders, faster response speeds, improved accuracy by focusing the image directly on to the photomultiplier tube and one-touch operation. Two photometer heads are available. With the selection of the correct peripherals, the system can be configured for the measurement of a.c., as single- and dual-emission systems and for microspectrophotometry. Two-dimensional software (Photoscan-2D) for life science distribution analysis and automatic measurement data processing software (Photoscan-E) are both run from external personal computers. In addition, a lightweight monochromator can be attached to the side of the photometer head for high-speed scanning of up to 400 nm s⁻¹ as required for qualitative spectral analysis.

■ Software

PlateCount from Taylor-Made Software is a menu-driven **program that tabulates microbiological viable count data** in a format that can be transferred electronically to standard statistical analysis programs (*Reader Service No. 110*). In addition, PlateCount calculates the number of ice nuclei associated with ice nucleation active bacteria, such as those that inhabit plant surfaces. The program is designed to save the microbiologist time by performing the calculations required to transform experimental results into a format that can be analysed statistically and should, the company says, be a useful tool to create on disk records of viable count or ice nucleation data. The \$100 (US) PlateCount program produces reports of both the raw data

entered and the calculated results. Reports can be printed or saved to a disk file.

Crystal Workbench from Molecular Simulations is a software package that combines the easy-to-use graphical user interface of the QUANTA molecular modelling system with the computational power of X-PLOR, a package for crystallographic refinement (*Reader Service No. 111*). This integrated solution provides WYSIWYG molecular modelling for X-PLOR. Crystal Workbench allows researchers to **develop modelling problems on desktop workstations** and then perform simulation calculations on any computing platform that supports X-PLOR, which includes platforms ranging from desktop systems to vector processing supercomputers and massively parallel machines. The program is also part of a family of products that includes NMR Workbench and Protein



Crystal Workbench-generated image.
High-resolution electron density map of Ribonuclease A with 3'-uracil monophosphate.

Workbench, making up a complete set of tools for macromolecular structure determination. Each of the workbench products is built upon Molecular Simulations' QUANTA molecular graphics analysis system, providing a single graphical user interface for all applications and all sizes of molecule being studied. QUANTA features interactive molecule construction and editing functions, extensive display and analysis capabilities and interfaces to all popular data formats. In NMR Workbench, these features are combined with a comprehensive spectral database for analysis of experimental NMR data. Protein Workbench features tools for sequence-directed protein folding and protein structural analysis. Crystal Workbench is available for workstation platforms from IBM, Hewlett Packard and Silicon Graphics, operating in both standalone or networked computing environments.

Gadgetry

Denley Instruments has designed a new **rotary plater** that allows bacteria to be plated out rapidly and with minimum spatter (*Reader Service No. 112*). Standard Petri dishes are placed on the electrically-driven turntable to produce either a spiral track of bacteria for colony separation techniques or

an even distribution of bacteria over the surface of the agar for phage-typing or antibiotic sensitivity testing. Denley also offers a range of handy gadgets for microbiology research, including an autospreader, a multipoint inoculator and autoclaves.

Measuring just 6 × 6 × 9 inches, the small Stat-Spin III **microcentrifuge** from Polyfionics features self-balancing to eliminate vibration and rapid acceleration and braking (*Reader Service No. 113*). The device can be fitted with a choice of rotors to accommodate two Eppendorf tubes, 12



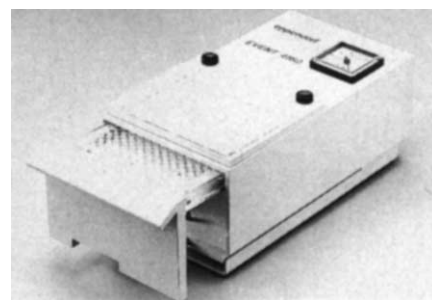
Stat-Spin III for fast spin downs.

haematocrit tubes or a special serum separator. When translated into spin times, users can perform plasma sample preparations in 30 s, HDL-cholesterol separations in 95 s and micro-haematocrits in 120 s.

For researchers using thin layer chromatography, Fungilab has introduced a new line of Fungicrom **separating chambers**

(*Reader Service No. 114*). Manufactured in glass, with flat floors and plain ground top edges, the Normaplak chamber can accommodate 200 × 200-mm TLC plates. The chamber is supplied with a lid that ensures a gas-tight seal. The Multiplak chamber has similar dimensions to that of the Multiplak but also has vertical grooves for holding up to five 200 × 200-mm TLC plates. The Miniplak chamber for 100 × 100-mm plates and the cylindrical Rollerplak chamber for 100 × 200-mm plates complete the line up. Fungilab can also supply chromatographers with the Jet Pak spray gun. The propellant gas is noncombustible and nonpoisonous and can apply a thin spray mist of reagent, the company says.

Unlike conventional **suction chambers**, the Event 4160 system from Eppendorf can be used anywhere in the laboratory: an integrated vacuum membrane pump makes it independent of compressed air installations and external vacuum pumps (*Reader*



Eppendorf's Event 4160 suction chamber.

Service No. 115). The Event 4160 can be used in immunological tests (for example, ELISAs), dot blots, filtration studies (for example, the separation of cells and media) and binding studies (for example, the binding of messenger substances to receptor molecules. For these and additional applications, Eppendorf supplies two types of

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Reader Service No.25

Event microtitre membrane plates: Event Detect and Event Cell.

Grade 2 water, the kind commonly used in analytical laboratories, traditionally has been produced by multiple distillation. According to Millipore, this process is both labour and energy intensive. Millipore now provides an alternative to multiple distillation in the Milli-U **water purification system**, which, the company says, is energy efficient and designed to keep operating and service costs to a minimum (*Reader Service No. 116*). An integral dispensing



Analytical-grade water on tap.

system attaches to most laboratory containers and automatically stops the filling process when the vessel is full. This set-up eliminates the need for large storage reservoirs and, therefore, the potential for contamination when water is stored for extended periods.

Process samples according to pre-set parameters with Heat Systems' **programmable ultrasonic homogenizer**, the XL-

2020 Sonicator, which is available from Labcaire Systems (*Reader Service No. 117*). The company says that the XL-2020 can be used to disrupt cells in seconds, extract enzymes, enhance chemical reactions, mix, homogenize, emulsify or de-gas samples before analysis, or even sonicate human immunodeficiency virus inside sealed tubes. The device provides 550 W of power in a compact, lightweight package. A tactile keypad allows the user to program into hard memory up to 10 different timing and/or pulsing functions, while an illuminated LCD screen provides user prompts, power monitoring and elapsed time displays. A wide variety of horns and accessories allow the user to process volumes from microlitres to litres.

■ Chromatography systems

LDC Analytical has developed a new **fluorescence detector**, the Model FM4100, which is designed to offer high degrees of sensitivity to facilitate the identification of unknown compounds and the detection of trace-level components (*Reader Service No. 118*). Analyses can be time-programmed and even automated for optimum throughput. The dual monochromator allows scanning of excitation and emission wavelengths to determine optimum wavelengths for each component. The FM4100 should be useful in the analysis of carbamates, PAHs, vitamins, amino acids, pharmaceuticals and environmental pollutants. The new detector is fully compatible with LDC's liquid chromatography Talk System, which offers full automation and integration. Alternatively, it can be operated as a standalone unit. All user interaction can be achieved through the front instrument panel with a membrane touch-sensitive keypad with LCD.

Ease of use is, says Chrompack, the hallmark of the company's new CP9001 **gas chromatograph** (*Reader Service No. 119*). A keyboard and 14-line display provide the user with easy control of GC samplers, satellites and accessories. Parameters can be

stored in up to 12 different methods. The method sequencing option allows the user to change from one method to another, when required, between injection. The Chrompack purge and trap system is an integrated system for the analysis of low concentrations (ppm/ppb) of volatile organic compounds in gaseous, liquid and solid matrices.

Konik Instruments has developed a **high-performance liquid chromatography system**, the HPLC 500 B, which, because it is modular, can be configured to suit individual needs (*Reader Service No. 120*). The standard version is fitted with a variable-volume injector. However, users can install different injection systems as well as an automatic column switching system, sample concentrator and repetitive injector. The column oven is standard in gradient models and optional in isocratic units. Four ultrafast electrovalves of minimum dead volume, controlled by the microprocessor, deliver the chosen percentages, previously selected, of any/or all four eluents in any combination and gradient. The system also features automatic priming and purging of the four solvent lines. Users can programme three external time events to control peripherals (autosamplers, column or detector switching valves, etcetera) and up to 10 complete methods can be stored on file in a protected memory. □

These notes are compiled by Diane Gershon from information provided by the manufacturers. For more information, fill in the reader service card bound inside the journal. Prices quoted are sometimes nominal and apply only within the country indicated.

ERRATUM

Nature 358, 521 (1992)

In the above product review, the detection sensitivity of trace impurities was given as in the parts-per-thousand range. It should have read, in the parts-per-trillion range.

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